# POSTSYNAPTIC ASPECTS OF NEUROGLANDULAR TRANSMISSION

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## ABSTRACT

Calcium ions have been found to produce a hyperpolarization of cockroach salivary gland acinar cells after a period of calcium deprivation and this 'readmission' response was prolonged by prior incubation in calcium-free media containing cobalt. The hyperpolarization was associated with an increase in membrane potassium permeability. Dopamine produced a hyperpolarization of acinar cells in the absence of calcium but consecutive responses to prolonged applications of the catecholamine were decreased and eventually abolished. The ionophore, A23187, evoked a large, prolonged hyperpolarization in calcium-containing solution and this, too, was associated with an increase in potassium permeability. In the absence of calcium, A23187 produced a small, transient response. The results suggested that the increase in potassium permeability was due to an increase in the intracellular calcium ion concentration and that dopamine and A23187 could evoke a hyperpolarization in calcium-free media by mobilization of calcium from a small, buffered, acinar store.

Dopamine has also been found to evoke responses on intracellular application.

Benztropine had no obvious effects on transmitter release or re-uptake but did show some inhibition of postsynaptic responses.

Dibutyryl cAMP had no effect on responses to dopamine and IBMX, a potent phosphodiesterase inhibitor, sometimes inhibited dopamine-evoked responses at high concentrations.

# INTRODUCTION

This thesis is a continuation of an electrophysiological investigation into the hyperpolarizing responses recorded from salivary gland acinar cells of the cockroach, Nauphoeta cinerea, Olivier. The main body of this work is concerned with elucidation of the role of calcium in the production of electrical responses in the acinar cells.

## Structure of the salivary glands

An excellent historical review of the elucidation of the structure of cockroach salivary glands has recently been given in a thesis by Bowser-Riley (1978a), a brief résumé of which will first be given for the convenience of the reader.

The first general description of the salivary gland of a cockroach was published in 1835 by Dufour. The gland was described as a bilateral structure lying on the ventral surface of the crop and comprised of groups of acini and of two reservoirs. Two pairs of ducts, one from the acini and one from the reservoirs joined to form the main salivary duct, which opened into the hypopharynx as first described by Huxley (1877). In 1858, Basch extended Dufour's work and described the acini as being composed of three layers - viz., an external envelope, an intimate membrane contiguous with the secretory ducts and a middle layer composed of granular, nucleated cells. Basch also initiated investigations into the biochemistry of saliva, showing that saliva converts

starch into sugar and this was confirmed by Plateau twenty years later. Bowser-Riley pointed out the importance of improvements in microscope design and of the development of histological techniques in the elucidation of the fine structure and innervation of the salivary glands. Thus, towards the end of the nineteenth century, Kupffer was able to distinguish two cell types, viz. peripheral and central, the peripheral cells being located directly beneath the basement membrane and between central cells. Kupffer also reported that the glands had two sources of innervation, one from the stomatogastric nervous system and the other from the ventral nerve cord; this was more extensively shown by Cholodkowsky (1881) and Hofer (1887). Lebedeff (1899), using histochemical techniques, observed that mucin was exclusively produced by the central cells and suggested that the peripheral cells produced a dilute and watery secretion. Thus, by the end of the nineteenth century, much of the general structure of the salivary glands of cockroaches had been described and an insight gained into the biochemical function of the cells (see Bowser-Riley, 1978a, for references in this paragraph).

More recently, with the aid of the electron microscope, in addition to the light microscope, the structure of the salivary glands of Periplaneta americana (Kessel and Beams, 1963) and Nauphoeta cinerea (Bland and House, 1971) have been investigated in more detail.

The peripheral cells are oval or pyramidal in shape and are in close proximity to the basement membrane. The intracellular ductule of one of these cells is pyramidal and, as it leaves the cell, narrows to become an efferent ductule which is continuous with the intercellular canaliculi and the lumen of the gland. These cells contain a single nucleus embedded in the cytoplasm and numerous mitochondria and ribosomes, but do not possess endoplasmic reticulum or secretory droplets - indeed, Bland and House suggested that these cells might be involved in the transport of ions and water. In contrast, the central cells, which form the bulk of the acinus, contain endoplasmic reticulum and large granules associated histochemically with secretion and, in particular, a mucoprotein. The central cells are joined by septate desmosomes and have microvilli near the junction of the cell with the beginning of the duct. Amylase, maltase, invertase and protease have been identified in extracts of Nauphoeta salivary glands and amylase production has been associated with the central cells. In an acinus, different central cells may be in different states of secretion at any one time; once release of the secretory material has occurred, there is a build-up of endoplasmic reticulum followed by formation of the dense secretory masses (Bland and House, 1971).

Duct cells near to the acini have large droplets of dense granular material and staining of Nauphoeta salivary

glands suggests the presence of a mucous component containing sialoglycans, these cells also containing a high tryptophan concentration. The major portion of the ducts, however, are composed of non-secretory cells which show much more invagination of the apical and basal membranes than do the secretory duct cells.

Innervation of the salivary glands

In 1970, Whitehead observed the innervation of the salivary glands of Periplaneta americana in electron micrographs. He reported that the pair of nerves from the suboesophageal ganglion contained two large axons about 5 μm in diameter and innervated the acini, ducts and reservoirs. The stomatogastric nerve from the stomodeal nervous system mainly innervates the crop (Willey, 1961) but a few fine branches associated with the salivary glands were observed to contain a dozen or more axons less than 1 μm in diameter. In 1971, Whitehead published a more detailed study of the salivary gland innervation, employing light and electron microscopy and histological techniques. The stomatogastric nerve of Periplaneta originates in the frontal ganglion, passes along the dorsal surface of the oesophagus and crop and, in the region of the salivary gland, branches are given off to anterior groups of acini. The paired salivary duct nerves, on leaving the suboesophageal ganglion, cross to the main salivary duct and follow it to its bifurcation. The nerves then pass along the reservoir ducts and, at the

mouths of the reservoirs, split into two large branches which divide further and innervate the acini and proximal areas of the reservoirs. When nerves reach an acinus they undergo much branching and ramify over the glandular surface forming plexuses. Branches of a nerve may cross between two or more acini forming a complex network and one acinus may be innervated from different nerves. The nerves either merge with the basement membrane thus penetrating extracellular spaces near central and peripheral cells or pass between central cells to deeper areas of the acinus. On the acinar surfaces the nerve fibres exhibit swellings but these do not appear to be associated with any specific cell structure.

The innervation of the salivary glands of Nauphoeta cinerea was described by Bowser-Riley (1978b), who employed light and electron microscopy in his study. He confirmed earlier reports that there were two sources of acinar cell innervation, the principal one being that arising from the suboesophageal ganglion. In Nauphoeta, a few branches of the stomatogastric nerve innervate acini lying adjacent to the reservoir but these nerves do not form acinar plexuses. In contrast to Periplaneta, Bowser-Riley found that, in Nauphoeta salivary glands, the salivary duct nerves send several branches to the acini before reaching the reservoirs. However, as in Periplaneta, much branching takes place over the acini forming plexuses, and varicosities are seen under the

basement membrane. Lateral groups of acini are innervated by branches of the ipsilateral duct nerve, while central groups of acini receive branches of both duct nerves. Three types of axons have been distinguished in Nauphoeta salivary glands by Maxwell (1978); one, the clear axon, contains no vesicular structures while the other two, named types A and B, do contain vesicles. Type A is five times as numerous as type B and contains large, dense vesicles (diameter, 920 Å) and smaller, agranular vesicles (440 Å). These axons are suggested to be catecholaminergic and are present between the basement membrane and cell membranes of peripheral or central cells; between central cells; between central cells and peripheral cells; and between central cells and duct cells. Type B axons contain large, densely packed vesicles (diameter 1380 Å) and are seen on the surface of cells underneath the basement membrane, but not between cells deeper in the acini.

Electrical responses

In 1973, House recorded hyperpolarizing responses from acinar cells on excitation of the salivary nerves by field stimulation. The resting potentials of the acinar cells varied between -14 and -70 mV, with a mean of -35 mV and hyperpolarizations varied in amplitude (from 1 to 30 mV) in response to a single shock. The responses had a latency of about 1 second, a time-to-peak of 2 seconds and a duration of about 10 seconds.

House also showed that the amplitude of the responses depended on the external potassium ion concentration. With regard to the different types of acinar cell, House later (1975) reported that hyperpolarizing responses were produced in both central and peripheral cells in response to nerve stimulation. The cells in an acinus are electrically coupled as shown by Ginsborg, House and Silinsky (1974), who stimulated the salivary duct nerves via a suction electrode into which was drawn the main salivary duct. They also presented evidence that the hyperpolarizing responses of the salivary gland were due, in the main, to an increase in potassium permeability.

Electrophysiological studies by Ginsborg and House (1976) showed that central acini were innervated from both ipsi- and contralateral salivary duct nerves, whereas lateral acini were innervated only by the ipsilateral nerve, and this was later confirmed by Bowser-Riley (1978b) in his morphological investigations. The stomatogastric nerve did not appear to make a contribution to the electrical responses, because these were observed to be identical whether produced by stimulation of the duct nerves alone or by field stimulation. It was also reported that the depolarization sometimes seen subsequent to the hyperpolarization was an independent response as it was not abolished by concentrations of α-flupenthixol which diminished the hyperpolarizing response.

Characterization of the neurotransmitter

Early pharmacological experiments involving insect salivary glands suggested a role for 5-hydroxytryptamine (5-HT), e.g. it induced secretion in Calliphora (Berridge and Patel, 1968) and Periplaneta (Whitehead, 1970) salivary glands. House (1973) also reported that 5-HT elicited hyperpolarization of Nauphoeta acinar cell membranes. A more involved investigation of locust salivary glands using histochemical and microspectrofluorimetric techniques suggested the presence of dopamine in the nerve fibres (Klemm, 1972).

Investigation of Nauphoeta salivary glands using microspectrofluorimetry indicated that a catecholamine rather than 5-HT was present in the nerve fibres and terminals (Bland, House, Ginsborg and Laszlo, 1973). Further characterization of the transmitter with the use of a radiochemical assay for simultaneous measurement of adrenaline, dopamine and noradrenaline established the presence of dopamine alone, each gland containing about 0.5 ng (Fry, House and Sharman, 1974).

Dopamine, adrenaline, noradrenaline and 5-HT all hyperpolarize acinar cells (Bowser-Riley and House, 1976) and induce secretion (Smith and House, 1977; House and Smith, 1978), dopamine being the most potent agonist. Hyperpolarization of the cells is not produced by a number of compounds, viz. α-aminobutyric acid, glutamate, glycine, aspartate, alanine, acetylcholine and carbachol

(Bowser-Riley and House, 1976); the α-agonists, amidephrine and methoxamine, and the β-agonist, isoprenaline (Ginsborg, House and Silinsky, 1976). A number of compounds related to dopamine have been tested on the cockroach salivary glands and those which hyperpolarized the acinar cells were found to be those previously reported to have effects on other systems acted on by dopamine (Ginsborg, Turnbull and House, 1976a). Both the electrical and secretory changes in the salivary glands are inhibited by phentolamine (Bowser-Riley, House and Smith, 1978) and by α-flupenthixol (House and Ginsborg, 1976; Breward, House and Smith, 1980) but propanolol, a β-receptor antagonist, has no effect on acinar cell hyperpolarization (Ginsborg et al, 1976). These pharmacological experiments suggest that the receptors in the cockroach salivary glands are not classical α or β receptors (Ahlquist, 1948) but that they bear similarities to dopamine receptors (Miller, Horn and Iversen, 1974; Ginsborg et al, 1976a).

Phentolamine is a competitive inhibitor of agonist-induced responses in the cockroach salivary glands. Measurement of its affinity constant using any of the catecholamines (adrenaline, noradrenaline and dopamine) gives a value of about $1\ \mu M^{-1}$, which is more than ten times that found using 5-HT as the agonist (Bowser-Riley et al, 1978). This suggests that 5-HT acts through different receptors from those acted on by the catecholamines.

Adrenaline, too, appears to mediate its effects through distinct receptors because the slope of the log dose-response curve for this agonist is much steeper than that for the other agonists (Bowser-Riley and House, 1976). A quantitative study of fluid secretion in response to simultaneous application of dopamine and noradrenaline showed an enhancement above that predicted of the two compounds acted on the same receptors (House and Smith, 1978). The disparity may be due to those agonists combining with different receptor binding sites but this remains to be satisfactorily resolved.

More recently, a comparison has been made of the time course of the hyperpolarizing response due to nerve stimulation with that of the response elicited by ionophoretic dopamine. There was found to be a strong similarity between the time courses of the two responses and both were affected to the same extent by a change in temperature (Blackman, Ginsborg and House, 1979a).

Thus the available evidence from all the investigations mentioned above points to dopamine as being the principal candidate for the role of neurotransmitter in the cockroach salivary glands.

## Cockroach salivary glands - fluid secretion and ion transport

Smith and House recently (1979) investigated the effects of changes in the concentrations of the ions sodium, potassium, chloride and calcium on the ionic

composition and volume of saliva secreted in response to 5-HT, dopamine and nerve stimulation. Saliva was collected from the cut end of a salivary duct into a pool of liquid paraffin for optical determination of its volume. Analysis of the ion content of saliva on stimulation of the glands by dopamine gave values of 121, 47 and 143 mM for sodium, potassium and chloride, respectively. A linear relationship was found between the rates of fluid secretion and sodium transport into the saliva suggesting that sodium transport provides the driving force for water movement. It was proposed that activation of receptors allows sodium influx (presumably related to the depolarization sometimes seen on stimulation) into the acinar cells and that a sodium-potassium pump on the luminal membrane extrudes sodium into the duct. In contrast, chloride ions appear to move across the acinar cells by passive diffusion because the rate of transport was found to be closely related to the external chloride concentration. A reduction in the potassium ion concentration of the bathing fluid reduces, but does not abolish, secretion and this was attributed to depression of the sodium pump. Calcium removal had little effect on dopamine-evoked secretion except in the presence of magnesium when consecutive responses to dopamine were markedly reduced. This decrease was rapidly reversed on readmission of calcium-containing solution, suggesting that these ions competed for a site involved in stimulus-secretion coupling.

Role of calcium in exocrine gland responses

The stimulation of fluid secretion in exocrine glands has, for some time, been linked with increases in ion movements (see Schneyer, Young and Schneyer, 1972). A possible role for calcium in the secretory responses of exocrine glands was proposed in 1963 when Douglas and Poisner showed that the stimulation of submaxillary gland secretion by acetylcholine and noradrenaline required the presence of calcium. This followed the report that calcium was required for acetylcholine-evoked catecholamine secretion from the adrenal medulla (Douglas and Rubin, 1961). Douglas (1968) proposed that influx of calcium into secretory cells was a prerequisite for secretion and was involved in extrusion of the contents of secretory granules from these cells. The importance of calcium in the regulation of the permeability of cell membranes of exocrine glands to other ions was established in 1971 by Batzri, Amsterdam, Selinger, Ohad and Schramm, who reported that the adrenaline-induced release of potassium ions from the rat parotid gland required calcium. This was associated with α-receptor stimulation, while β-receptor stimulation induced enzyme release, probably via cyclic - 3':5' - adenosine monophosphate (cAMP) formation (Batzri, Selinger and Schramm, 1971). Later experiments employing the calcium ionophore A23187 suggested that potassium was released in response to an increase in the intracellular calcium ion concentration

(Selinger, Eimerl and Schramm, 1974). It was proposed that adrenaline acted on α-receptors to stimulate calcium influx and this was confirmed by $^{45}Ca$ uptake studies (Kanagasuntheram and Randle, 1976).

Further experiments on the calcium requirement of salivary glands for potassium release were performed by Putney and co-workers, who measured radioactive rubidium release from loaded parotid slices as a marker for potassium efflux. It was shown that activation of muscarinic, α-adrenergic and peptide receptors resulted in $^{86}Rb^{+}$ release from preloaded slices and that the release consisted of an early transient phase, independent of the presence of external calcium, and of a sustained, calcium-dependent phase (Putney, 1976, 1977). It was proposed that the transient phase was due to mobilization of calcium from a store which was inaccessible to EGTA. It was also proposed that the three receptors activated the same calcium influx sites because an agonist for one of the receptors did not elicit a transient response in calcium-free medium if there had been a prior exposure to an agonist for one of the other receptors (Putney, 1977). Further evidence for a common store of calcium came from experiments where agonists of different receptors were used alone or in combination and no summation of transient or sustained phases was seen when two agonists were applied together (Marier, Putney and Van de Walle, 1978). Electrophysiological experiments

in the parotid gland showed that muscarinic and α-adrenergic agonists elicited hyperpolarization of the membrane probably due to an increase in the potassium permeability (Pedersen and Petersen, 1973; Petersen and Pedersen, 1974). In calcium-free medium the adrenaline-induced hyperpolarization was still seen, presumably due to release from intracellular stores, but there was a shortening of the response after repeated stimulation (Petersen and Pedersen, 1974).

Calcium regulation of potassium permeability has been reported in other exocrine glands, e.g. the submaxillary gland (Martinez and Quissell, 1976; Martinez, Quissell and Giles, 1976) and the lacrinal gland (Parod and Putney, 1978). Electrophysiological studies of the effects of acetylcholine in these glands suggest that there is an increase in permeability to sodium in addition to the increase in potassium permeability but any role for calcium in these changes has not been extensively investigated (Nishiyama and Petersen, 1974; Iwatsuki and Petersen, 1978a). More detailed studies implicating calcium in activation of membrane sodium permeability have been performed on pancreatic exocrine tissue. Acetylcholine induces depolarization of pancreatic acinar cells, and increases sodium, chloride and potassium permeabilities but there is no evidence for an increase in permeability to calcium ions (Iwatsuki and Petersen, 1977a). Depolarization of pancreatic cells is also

induced by bethanechol, an effect which is still observed in calcium-free medium. However, A23187 induces a calcium-dependent depolarization, which is also dependent on sodium suggesting that an increase in the intracellular calcium ion concentration increases the sodium permeability (Poulsen and Williams, 1977). The importance of intracellular calcium in the depolarization of the cells was further shown by intracellular calcium ion injection (Iwatsuki and Petersen, 1977b). The depolarizations seen in calcium-free medium to acetylcholine and bethanechol may be explained by assuming that these secretagogues mobilize intracellular calcium stores to increase the cytosolic calcium concentration which mediates a membrane permeability change to sodium in these glands.

Calcium and the blowfly salivary glands

The most thoroughly investigated invertebrate salivary gland in relation to the effects of calcium is that of the blowfly Calliphora. In contrast to the acinar structure of mammalian and cockroach salivary glands, the blowfly gland consists of two closed, elongated tubes containing only one cell type (Oschman and Berridge, 1970). 5-HT induces changes in the transepithelial potential and its effect on this potential reflects changes in the apical membrane potential rather than in the basal membrane potential (Prince and Berridge, 1972). Prolonged superfusion of 5-HT elicits a prolonged increase in negativity of the transepithelial potential and this

effect is dependent on the presence of chloride ions (Berridge and Prince, 1972). However, consecutive, short exposures to the agonist result in biphasic potential changes, the potential becoming more positive than the resting level after an initial increase in negativity. Berridge and Prince suggested that cAMP mediated the action of 5-HT in production of the positive potential (i.e. hyperpolarization of the apical membrane) via activation of a potassium pump. These authors also proposed that the negative potential change was evoked by a calcium-dependent increase in the apical membrane permeability to chloride ions (Prince and Berridge, 1973). Consistent with the latter hypothesis is the finding that 5-HT stimulates uptake of calcium ions into gland cells (Prince, Berridge and Rasmussen, 1972) and that the calcium ionophore A23187 induces a negative potential change in the presence of chloride ions (Prince, Rasmussen and Berridge, 1973). The transepithelial resistance is decreased by 5-HT and this change is calcium and chloride dependent (Berridge, Lindley and Prince, 1975); also, the initial negative potential change but not the positive change may be reversed by altering the membrane potential, and these observations are consistent with the hypotheses, above, for 5-HT action.

The prolonged increase in fluid secretion from the blowfly salivary gland in response to 5-HT and A23187 is dependent on calcium (Prince et al, 1972, 1973).

However, in the absence of calcium these compounds can induce fluid secretion for a limited period presumably due to mobilization of an intracellular calcium store (Prince and Berridge, 1973; Berridge, 1975). Chloride movement appears to facilitate fluid secretion as the secretory response to cyclic AMP is much smaller when the chloride ion concentration is reduced (Prince and Berridge, 1973). Presumably at normal chloride concentrations, cyclic AMP releases calcium from intracellular stores to promote chloride secretion (Prince et al, 1972) and, hence, an increased flow of saliva.

More recent investigations by Fain and Berridge (1979a) suggest a relationship between the hydrolysis of phosphatidylinositol and 5-HT mediated calcium uptake into Calliphora salivary glands. Such a mechanism for the gating of calcium has been proposed in a number of tissues, including mammalian exocrine glands (Michell, 1975; Jones, Cockroft and Michell, 1979). The release of {$^{3}$H} inositol from phosphatidylinositol in the blowfly salivary gland on application of 5-HT occurred to the same extent in the absence, as in the presence of calcium, while secretion was reduced. The ionophore A23187 had no effect on inositol release in concentrations which evoked fluid secretion. These observations are consistent with the hypothesis that 5-HT stimulates phosphatidylinositol breakdown as a prelude to calcium uptake. 5-HT also inhibits phosphatidylinositol synthesis

in the presence of calcium (Berridge and Fain, 1979). Prolonged stimulation by 5-HT in the presence of calcium resulted in a gradual decrease (after 2 hours) of $^{45}Ca^{2+}$ uptake. This uptake could be reactivated by incubation in inositol suggesting that inactivation of calcium transport was linked to a decrease in phosphatidylinositol concentration. No change was detected in the total concentration of phosphatidylinositol although 80% of the labelled compound was broken down (Fain and Berridge, 1979b) and it was proposed that the pool of phosphatidylinositol subject to labelling and concerned with calcium gating represented only a small fraction of the total phosphatidylinositol concentration in the blowfly salivary gland.

Cockroach salivary glands - ions and the electrical responses

The effects of changes in the potassium ion concentration on the electrical responses of the salivary glands have indicated that the hyperpolarizations recorded on stimulation of the glands are due mainly to an increase in the membrane potassium permeability (Ginsborg et al, 1974). Further investigation of the mode of action of dopamine is warranted and, in view of the importance of calcium in mammalian and invertebrate salivary gland responses, the present study was undertaken to determine the role of this ion in the electrical responses of the cockroach salivary glands (Section I).

In addition (Section II) three short open-ended investigations are reported. These are concerned with

i) receptor localization,

ii) the effects of benztropine, which has been proposed either as a dopamine uptake inhibitor or as a dopamine releasing agent,

iii) the role of adenylate cyclase.

# GENERAL METHODS

Cockroaches of the species Nauphoeta cinerea, Olivier, were reared for me in glass aquaria and provided with rat cake and water ad libitum. The salivary apparatus, on removal from the cockroach, was routinely bathed in a solution of composition (mM): NaCl, 160; KCl, 1; $CaCl_2$, 5; Tris-(hydroxymethyl)-methylamine HCl buffer pH 7.6 - 7.8, 5. This was the standard solution used in the dissection and in most experiments and modifications are as specified in the text.

Dissection

A cockroach (male or female) was pinned, ventral side down, through the head and the tip of the abdomen onto silicone resin (Sylgard 184, Dow Corning) in a shallow perspex dish. The antennae, legs and wings were removed and the insect was immersed in solution (see above). One of the abdominal segments was raised and the tissue ventral and lateral to this was cut. The dorsal segments were dissected away at their lateral edges and the tracheae and fat below were removed to expose the internal organs. The gut was pinned out to the side to reveal the salivary apparatus lying along the ventral side of the gut. The glands were freed of two tracheal branches and the reservoirs were held away from the gut with forceps so that the connective tissue holding the glands to the crop and oesophagus could be cut. The labium and first few ventral segments were removed together with some white fat to expose the main salivary duct. The ventral nerve cord

was gently pulled away from the head to expose the suboesophageal ganglion, and connections to the salivary glands were severed. After transection of the main salivary duct, the freed salivary structures were transferred to bathing solution in the experimental chamber and pinned out, slightly stretched, over a dome of Sylgard.

Experimental design

Figure 1 shows a diagram of the experimental chamber (volume, 3 ml), which was constructed from a perspex block. The base of the chamber was covered with a layer of Sylgard resin and, once this had solidified, a dome was made in the centre using drops of slightly solidified resin and allowed to set. The preparation was pinned onto the Sylgard so that the acini were held against the dome, thus preventing movement in the circulating fluid. The glands were perfused (usually at 2 ml $min^{-1}$) with solution pumped continuously by a Watson Marlow H.R. Flow inducer and turbulence in the bathing fluid was minimised by the placement of nylon mesh between the preparation and the inflow and outflow tubes.

The bath was mounted on a rigid table and the preparation viewed through a Zeiss dissecting microscope (x 80). The whole apparatus was enclosed in an earthed Faraday cage to protect against electrical interference.

The basic experimental layout for stimulation and recording is shown in Figure 2. The suction electrode

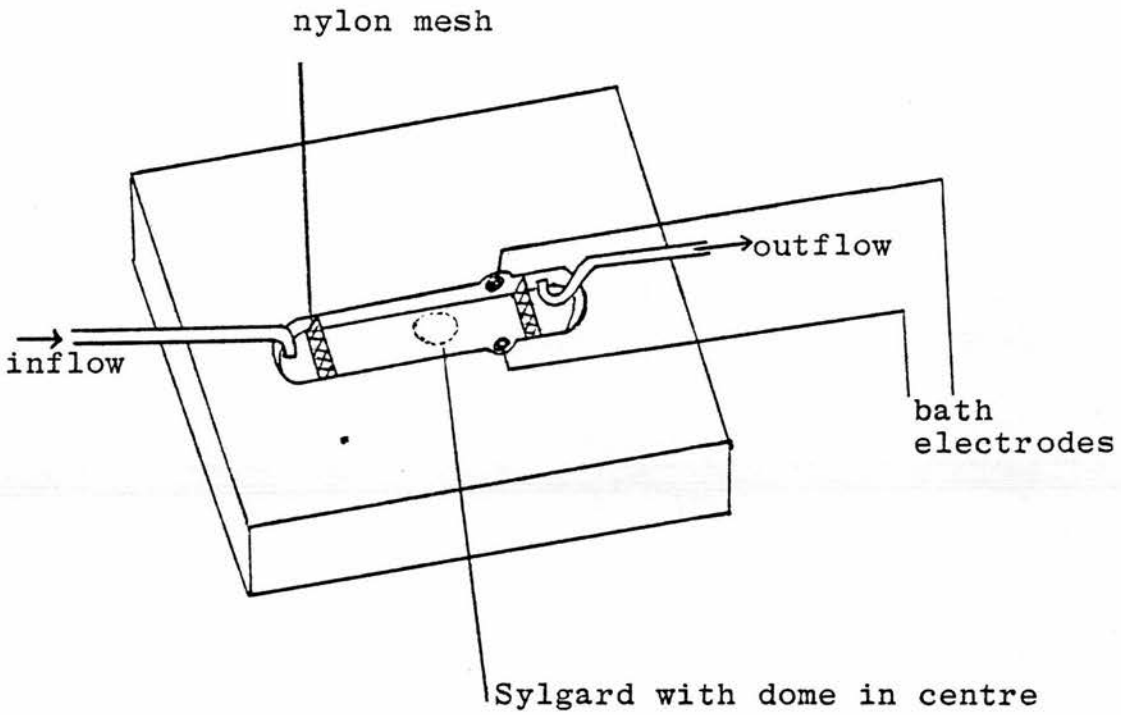



FIGURE 1: Diagram of experimental chamber.
See text for details.

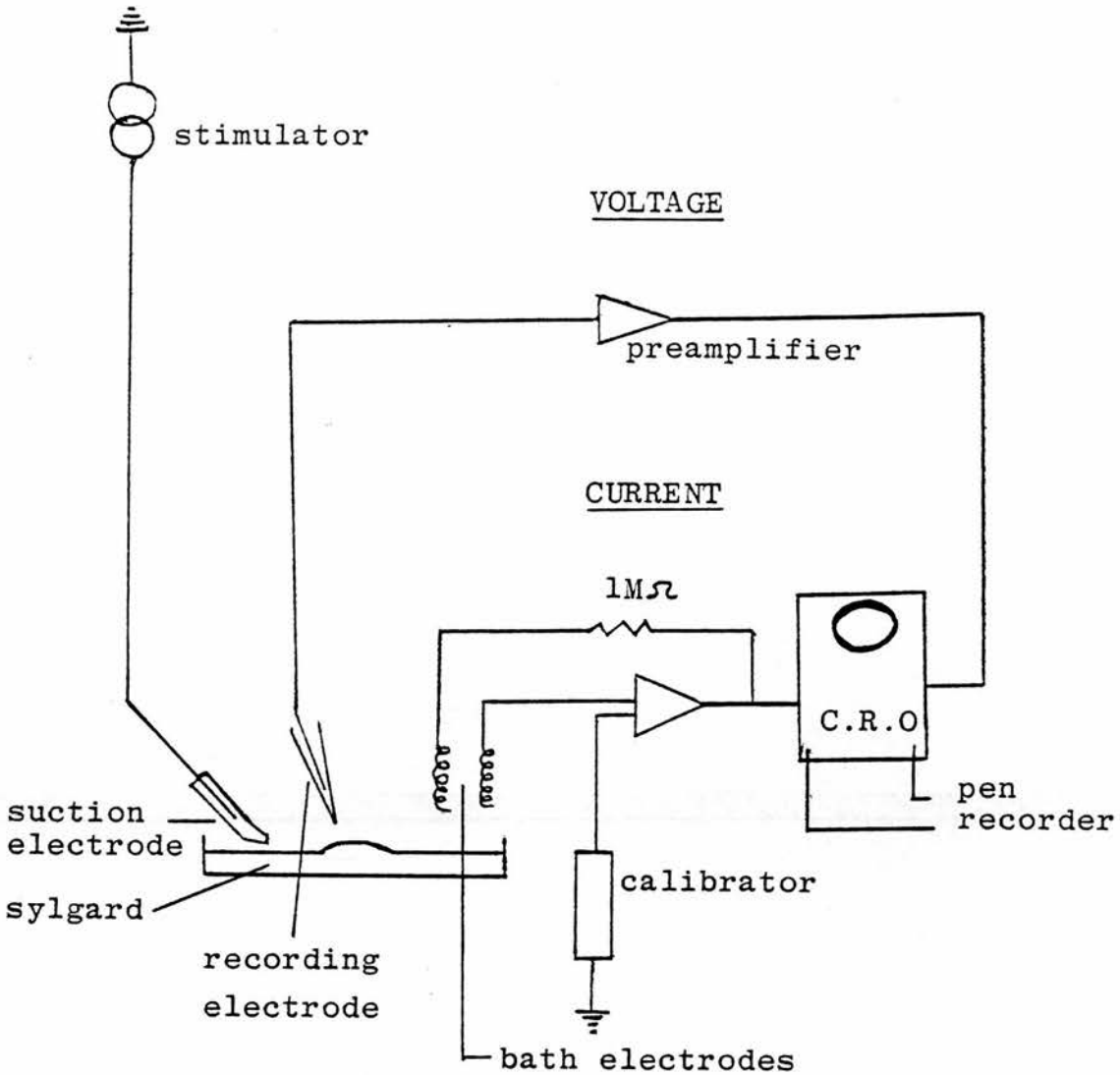



FIGURE 2: Diagram of the apparatus used for stimulation and recording.

was constructed from a rigid polythene tube, one end of which was drawn out so that the aperture was similar in size to the diameter of the main salivary duct to allow a close fit around the duct. A syringe was attached by a cannula to the other end to allow bathing fluid and the salivary duct (with the salivary nerves) to be sucked into the electrode, in which was the anode, the cathode being present on the outer surface and immersed in solution. This suction electrode was used to stimulate the salivary nerves by a single pulse or short trains (2 - 20 at 100 $H_3$) of pulses (0.5 ms, 10 - 50 V) from a square pulse stimulator (Devices, Type 2533). The frequency of application of a stimulus to the nerves (or to an ionophoretic electrode) could be controlled automatically using a Devices Digitimer.

Membrane potentials were recorded with glass microelectrodes made on a vertical electrode puller from borosilicate glass capillaries (outer diameter, 2 mm), containing an inner filament to aid filling (Clark Electromedical Instruments, EC200F - 15). These electrodes (10 - 20 Mn resistance) were filled with 1 - 3M KCl using a syringe fitted with a fine needle, and were connected to a dual-beam oscilloscope (Tektronix, Type 565) via a high impedance preamplifier (WP Instruments, dual micro-probe 750). These microelectrodes were lowered into position by means of a Zeiss sliding micromanipulator and placed close to an acinus, which was penetrated by using the fine control on the micromanipulator or by giving a light tap to the table on which the apparatus rested.

The bath electrodes consisted of AgCl - coated Ag wire and were mounted as shown in Figure 1. The circuit for monitoring current (Figure 2) was similar to that described by Gage and Isenberg (1969) using an operational amplifier (E - 78 differential amplifier, Computing Techniques Ltd.) connected in the configuration as suggested by Moore (1963), and incorporated a D.C. calibrator (Electrophysiological Instruments Ltd.). The 1 MΩ resistor connected in parallel with the operational amplifier afforded a 1 nA output to the oscilloscope for a 1 mV input. Permanent records of voltage and current were obtained using a pen recorder (Gould Brush 220).

Dopamine was applied ionophoretically by giving pulses (up to 1 μA for up to 1 second) from a Devices stimulator (Type 2533) to micropipettes (5 - 15 MΩ resistance) containing a solution (0.25 - 1M) of dopamine HCl (Koch-Light). Backing current of 5 - 7 nA was applied to decrease spontaneous leakage of dopamine from the pipettes.

Conductance changes in an acinus were measured by injecting rectangular current pulses (100 nA) through an intracellular electrode and recording electronic potentials with a second microelectrode in the same acinus.

Any additions or modifications to the above methods are described in the relevant sections.

# SECTION I

## A STUDY OF THE ROLE OF CALCIUM IN THE ELECTRICAL RESPONSES OF THE COCKROACH SALIVARY GLANDS.

## Introduction

This section is a report of an investigation into the involvement of calcium in hyperpolarizing responses recorded from acinar cells. The hyperpolarization has been found to be due to calcium-dependent potassium activation (see Meech, 1978) and it is proposed that the action of dopamine initially mobilizes calcium from an acinar store, which may be replenished from the external fluid.

The investigation is divided into three parts:

Part 1 shows that calcium can produce a hyperpolarization of acinar cells and relates this to an increase in potassium permeability.

Part 2 gives evidence for a buffered calcium store which may be 'topped up' with calcium from the bathing solution and which is linked with the action of dopamine.

Part 3 employs a calcium ionophore to bypass the initial step of receptor activation and provides further evidence for calcium-dependent potassium activation.

# SECTION I: Part 1

## A calcium readmission response recorded from acinar cells and the effect of cobalt treatment.

## Introduction

It was first reported by Douglas and Rubin (1961) that calcium readmission after a period of deprivation resulted in secretion from adrenal medullary cells and it was suggested that this was due to an increase in calcium permeability in calcium deficient medium. In the following experiments on the salivary glands, readmission of calcium after a period of incubation in calcium-free solution is shown to evoke a hyperpolarization which is related to an increase in potassium permeability arising from an increase in the intracellular calcium ion concentration. Such calcium-dependent potassium activation was initially described in erythrocytes (Gardos, 1958; Romero and Whittam, 1971) and has since been observed in many nervous tissues (see Meech, 1978, for review).

Cobalt, like magnesium, is a competitive inhibitor of calcium at motor nerve terminals (Weakly, 1973) and both ions have been used in these experiments to substitute for calcium in calcium-deficient media. The antagonism by cobalt of calcium-dependent effects has been demonstrated in a number of preparations, e.g. blockade of synaptic transmission in cerebellum (Hackett, 1976) and in sympathetic ganglia (Guerrero and Riker, 1973); inhibition of $Ca^{2+}$ influx associated:

1. with action potentials in the barnacle muscle fibre (Hagiwara and Takahashi, 1967) and in Aplysia neurones (Geduldig and Junge, 1968);

2. with the after-hyperpolarization in spinal motoneurones (Krnjević, Lamour, MacDonald and Nistri, 1978); and

3. with the slow inward current in squid axons (Baker, Meves and Ridgway, 1973) and in mammalian myocardial fibres (Kohlhardt, Bauer, Krause and Fleckenstein, 1973).

Cobalt also inhibits hormone release from the neurohypophysis (Dreifuss, Grau and Nordmann, 1973) and pancreatic β-cells (Henquin and Lambert, 1975).

These effects are generally believed to result from inhibition of calcium entry into cells. However, other effects of cobalt, e.g. inhibition of fast axonal transport (Hammerschlag, Chiu and Dravid, 1976) and spontaneous neurotransmitter release (Weakly, 1973) have been attributed to calcium displacement from intra-cellular binding sites by cobalt.

Cobalt treatment in the salivary glands is shown to prolong the hyperpolarization on calcium readmission and it is suggested that this arises from a prolonged increase in calcium permeability caused by cobalt.

METHODS (see also General Methods)

In the experiments in this section there was no addition of $CaCl_2$ to the 'conditioning' solutions and some contained 5 or 10 mM $MgCl_2$, 1 mM $Mg^{2+}$ + 1 mMEGTA, or 5 mM $CoCl_2$ (see text).

Input resistance was measured by injecting rectangular current pulses through an intracellular electrode and recording electrotonic potentials with a second microelectrode in the same acinus. The reversal potential of the readmission response may be calculated from the electrotonic potentials observed in conditioning and readmission solutions. In Figure 1A the applied current pulse (I) is constant throughout the experiment and the resulting electrotonic potentials have magnitude P before and p after the readmission of calcium. The hyperpolarization obtained with zero current has magnitude v and is of the same sign as the resting potential, E, in the conditioning solution. Assuming a circuit as shown in Figure 1B (see Katz, 1966), the readmission response is represented by closing of the switch. The resistances R and r are shown and the reversal potential is e, where

$$e = E + \left(\frac{P}{P - p}\right) v$$

(see Figure 1, calculation)

RESULTS

On replacement of the standard bathing solution (containing 5 mM calcium) with a 'conditioning' solution without added calcium a small depolarization (of up to 10 mV) was sometimes seen (Figure 4A). When the calcium-containing solution was readmitted a large hyperpolarization was elicited (Figures 2 - 4). In the experiment of Figure 2

FIGURE 1: Calculation of the reversal potential for the readmission response. See text for definition of symbols in A and B opposite.

also, conductances $G = \frac{1}{R}$ and $g = \frac{1}{r}$

$V$ = potential across each arm of the circuit

$i_1$ = current through left arm

$i_2$ = current through right arm

$V_0$ = potential in conditioning solution (i.e. with an open switch in the circuit).

$V = E + i_1R$ and $V = e + i_2r$ ; also, $i_1 + i_2 = I$

Combining :

$$\frac{V - E}{R} + \frac{V - e}{r} = I \quad \text{(switch closed)....(1)}$$

$P = \frac{I}{G}$ and $p = \frac{I}{G + g}$ so, $g = \left(\frac{P - p}{p}\right)G$ .....(2)

From (1) and (2) $V = \frac{I + GE + ge}{G + g}$

With switch open, $V_0 = \frac{I + GE}{G}$

Change in potential when switch is closed = $V - V_0$

$$V - V_0 = \frac{\frac{g}{G}(e - V_0)}{1 + \frac{g}{G}}$$

When $I = 0$, $v = V - V_0$ so, $v\left(\frac{G}{g} + 1\right) = e - E....(3)$

From (1) and (3) the reversal potential, $e$ , is found :-

$$e = E + \left(\frac{P}{P - p}\right)v$$

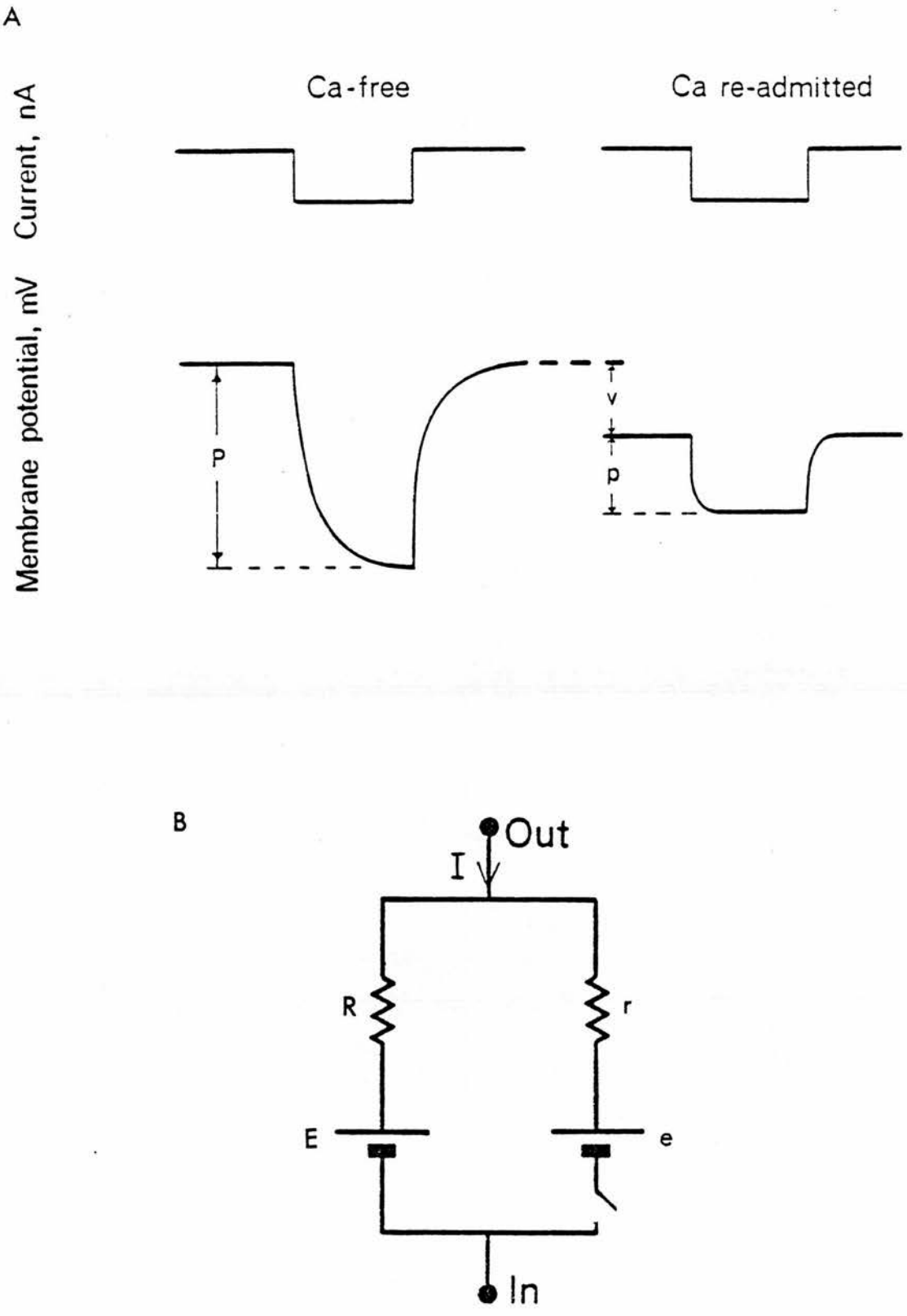



FIGURE 1: See text and calculation (opposite).

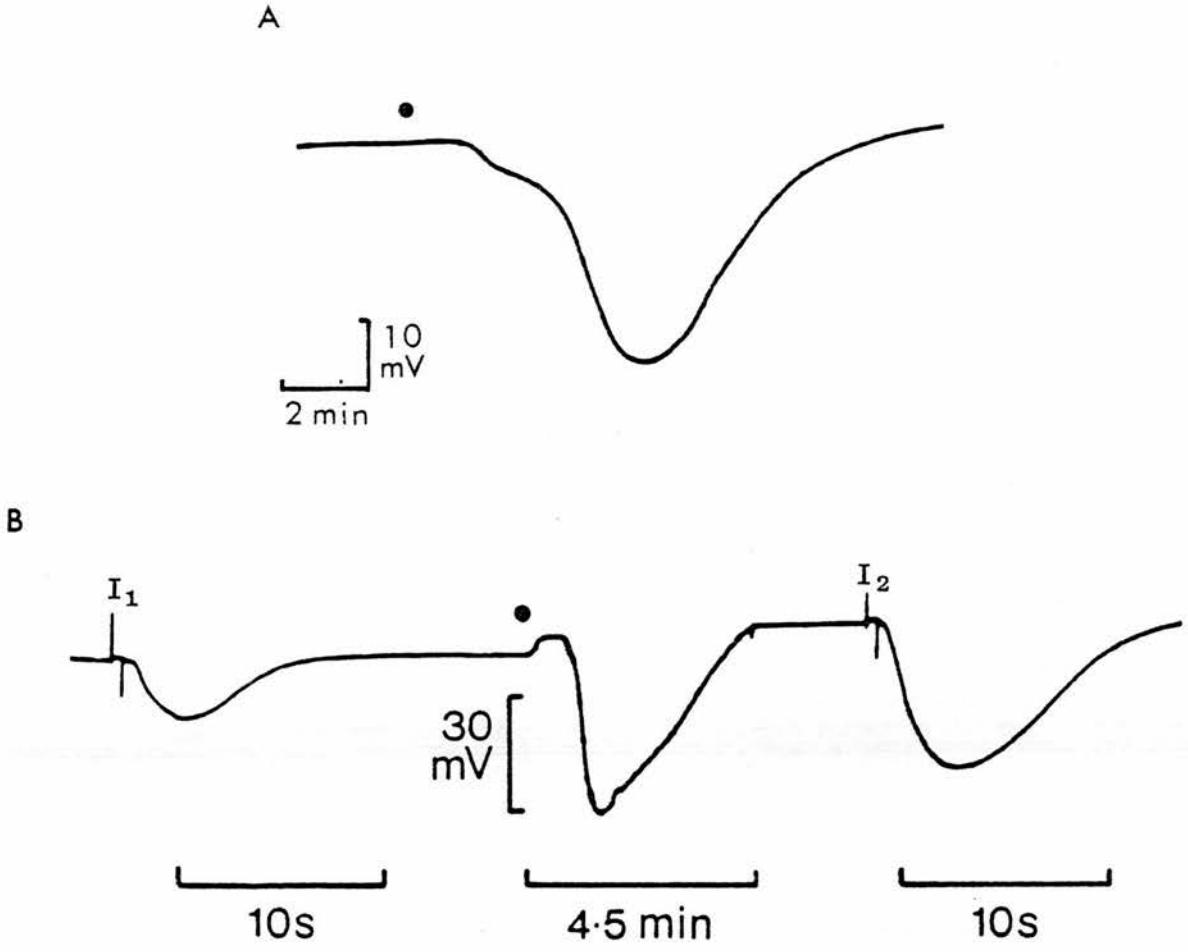



FIGURE 2: Readmission responses after incubation in media with no added divalent cations.

In A, the preparation was incubated in calcium-free solution containing 1 mM EGTA for 10 min; in B, the preparation was incubated in calcium-free medium for 15 min. At • the glands were exposed to control media containing 5 mM calcium.

In B, $I_1$ and $I_2$ are responses to identical pulses of dopamine in 'conditioning' and 'control' solution respectively.

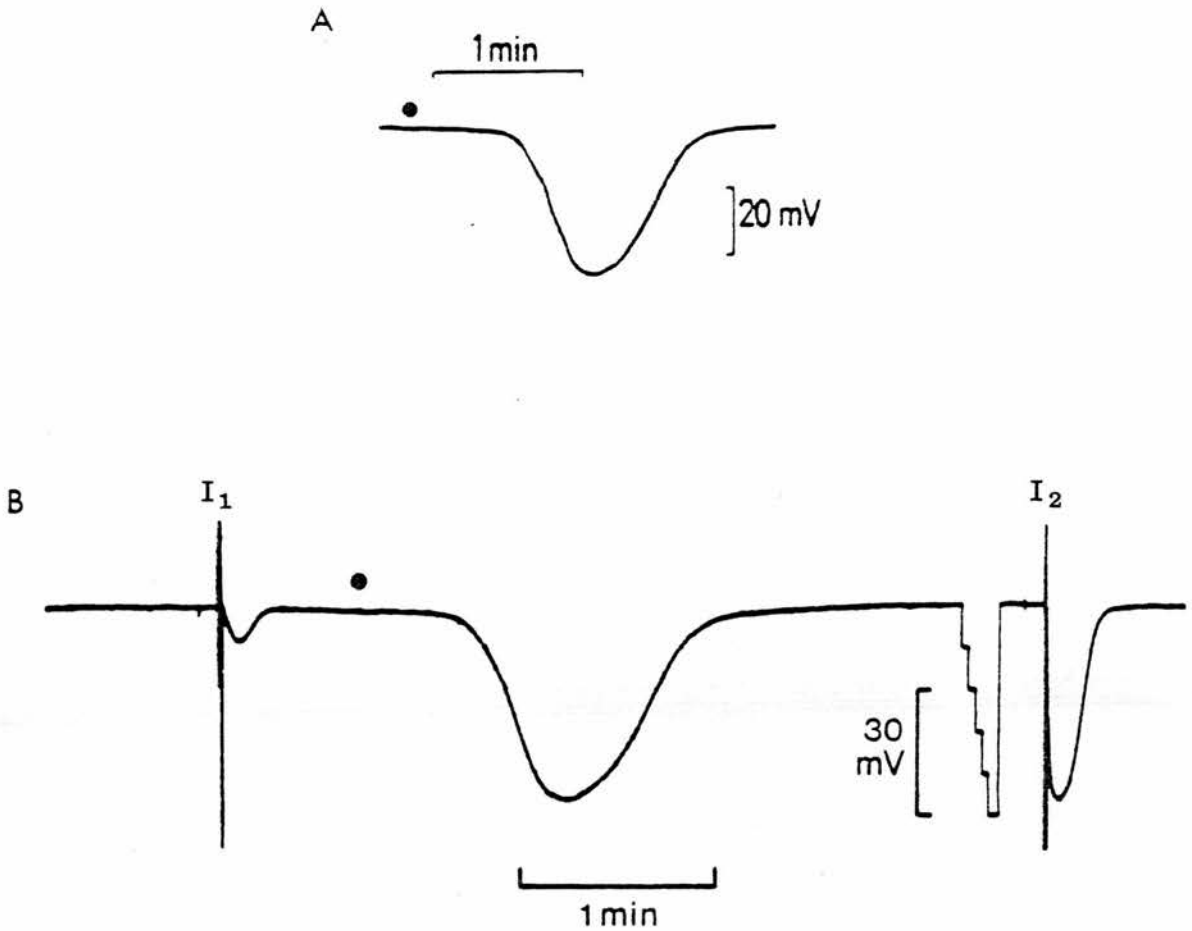



FIGURE 3: Readmission responses after incubation in calcium-free media containing magnesium.

In A, the glands were incubated in solution containing 5 mM $MgCl_2$ for 13 min; in B, the glands were incubated in solution containing 10 mM $MgCl_2$ for 16 min. Control solution containing 5 mM $CaCl_2$ was introduced at •.

In B, the responses, $I_1$ and $I_2$ to identical pulses of dopamine were elicited in conditioning and in control solutions respectively.

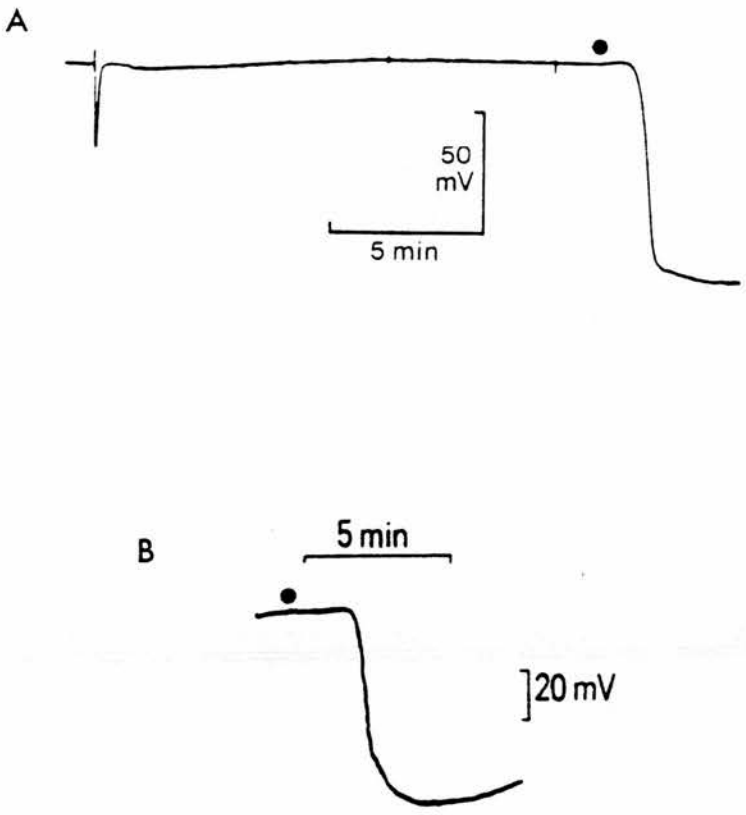



FIGURE 4: Readmission response after incubation in zero calcium medium containing cobalt.

In A, control solution containing 5 mM $CaCl_2$ was replaced by calcium-free solution containing 5 mM $CoCl_2$ shortly after a response was evoked by a stimulus to the salivary nerves. Control solution was reintroduced at • after 15 min in the cobalt medium.

In B, the preparation was incubated in medium containing 5 mM $CoCl_2$ + 5 x $10^{-4}$M phentolamine. At • control solution was reintroduced.

no divalent ions were present in the conditioning solutions and readmission of calcium resulted in transient hyperpolarizations. Transient readmission responses were also seen if the conditioning solution contained magnesium (Figure 3), but incubation in cobalt containing solution resulted in production of a prolonged hyperpolarization (Figure 4).

It was not always possible to determine the duration of the response after cobalt incubation because of displacement of the recording microelectrode but Table 1 gives an indication of the more prolonged nature of the response elicited after cobalt treatment than that produced after incubation in solution containing magnesium or no added divalent ions. In order to investigate the readmission response more fully it was therefore of benefit to incubate the preparation in conditioning solution containing cobalt.

It was found that the hyperpolarization after cobalt treatment was sustained only if calcium was present in the bathing solution and was reversed by replacement of the calcium containing solution with one containing cobalt (Figure 5) or magnesium (Figure 6). The membrane potential reverted to the hyperpolarized level on readmission of the calcium solution.

TABLE 1: Representative selection of readmission responses

| Divalent cations in conditioning solution (mM) | Duration of exposure to conditioning solution (min) | Amplitude of hyperpolarization on readmission of 5 mM-calcium (mV) | Duration of response (time to return to within 5 mV of original membrane potential) (min) |
|---|---|---|---|
| None | 9 | 40 | 5 |
| | 27 | 20 | 9 |
| | 40 | 70 | >9 |
| None (+ 1 mM-EGTA) | 9 | 30 | >2 |
| $Mg^{2+}$ (1) (+ 1 mM-EGTA) | 17 | 40 | 2 |
| | 20 | 65 | 3 |
| | 43 | 20 | 2 |
| $Mg^{2+}$ (5) | 40 | 45 | 2 |
| $Co^{2+}$ (2.5) | 35 | 80 | >30 |
| | 9 | 80 | >30 |
| | 10 | 70 | 8 |
| | 15 | 55 | >40 |
| $Co^{2+}$ (5) | 17 | 70 | >15 |
| | 18 | 60 | >120 |
| | 20 | 65 | >4 |
| | 25 | 45 | >90 |
| $Co^{2+}$ (5) (+ 1.5 mM-phentolamine) | 10 | 70* | 19 |
| | 16 | 65* | >16 |
| Mean ± SE of mean | - | 55 ± 5 | - |

* Readmission solution also contained 1.5 mM-phentolamine

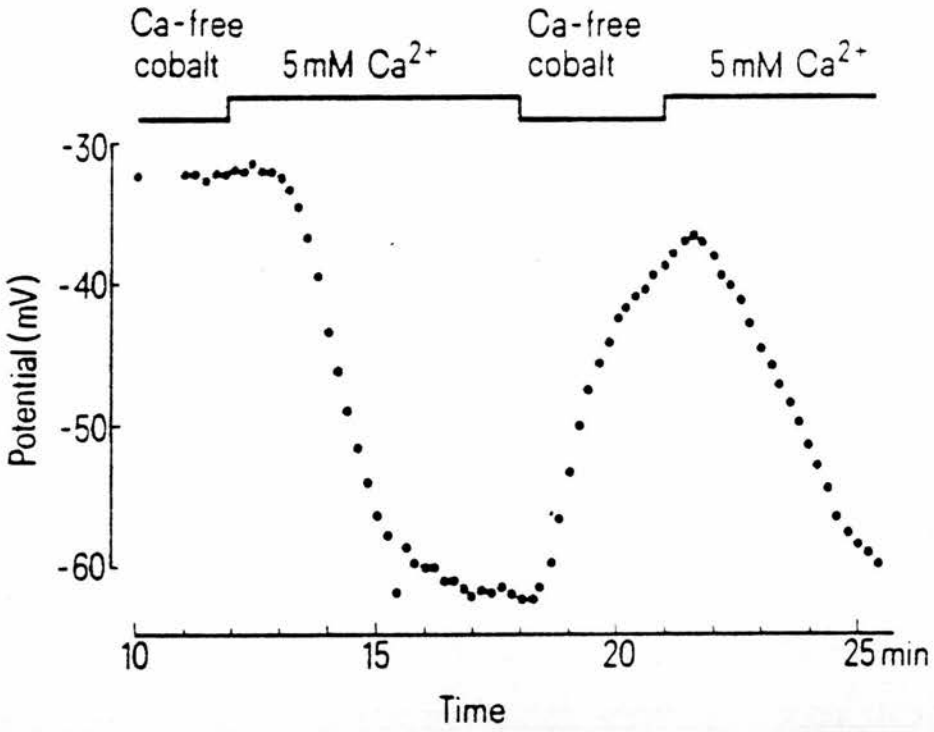



FIGURE 5: Effect of withdrawal of calcium on the readmission response.

Abscissa: time after start of exposure to 5 mM cobalt.

Ordinate: membrane potential of acinar cell.

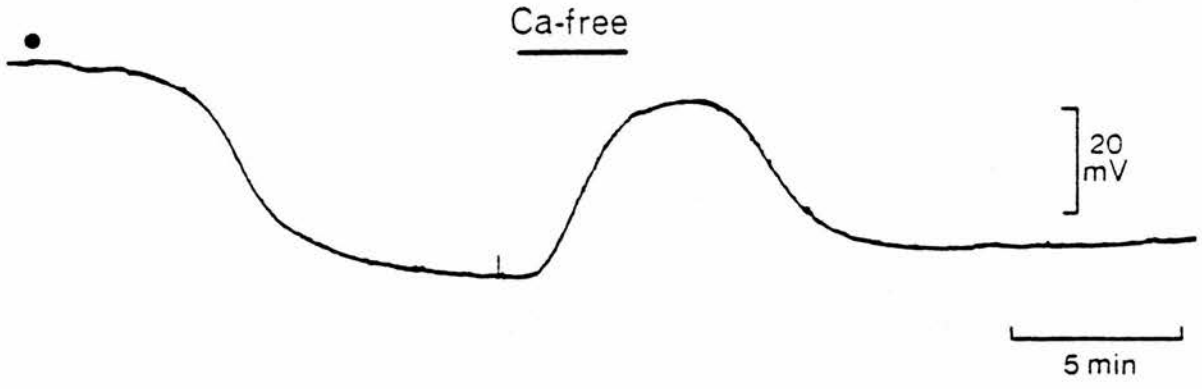



FIGURE 6: Effect of calcium withdrawal on the readmission response.

The preparation was incubated in calcium-free medium containing 5 mM cobalt for 25 min before control solution containing 5 mM calcium was introduced (at •). During the period shown by the horizontal bar the control solution was replaced by calcium-free medium containing 10 mM magnesium.

Modification of 'conditioning' and 'readmission' solutions

Variations in the composition of the conditioning or readmission solutions affected the magnitude of the readmission response.

In Figure 7 the preparation was incubated for 30 minutes in 0.5 mM cobalt and reintroduction of 5 mM calcium produced a hyperpolarization of 20 mV. The preparation was then exposed to 5 mM cobalt for 14 minutes, during which time the hyperpolarization was reversed. Reintroduction of 5 mM calcium was seen to produce a hyperpolarization significantly greater (over 60 mV) than that elicited after incubation in low (0.5 mM) cobalt.

In Figure 8 the preparation was exposed to 5 mM calcium together with 5 mM cobalt and washout of the cobalt solution resulted in only a small hyperpolarization (7 mV). Incubation in 5 mM cobalt alone for a further 25 minutes was followed by a large hyperpolarization (60 mV) on return to the calcium solution.

Figure 9 shows that the magnitude of the readmission response was dependent on the calcium concentration, a much greater response being elicited by readmission solution containing 5 mM calcium than one containing 1 mM calcium. Reversal of the hyperpolarization occurred in solution containing a low (0.1 mM) calcium concentration (Figure 10) as was observed in zero calcium solutions containing cobalt or magnesium (Figures 5 and 6). The presence of 5 mM cobalt in the readmission solution also reduced the magnitude of the hyperpolarization (Figure 11).

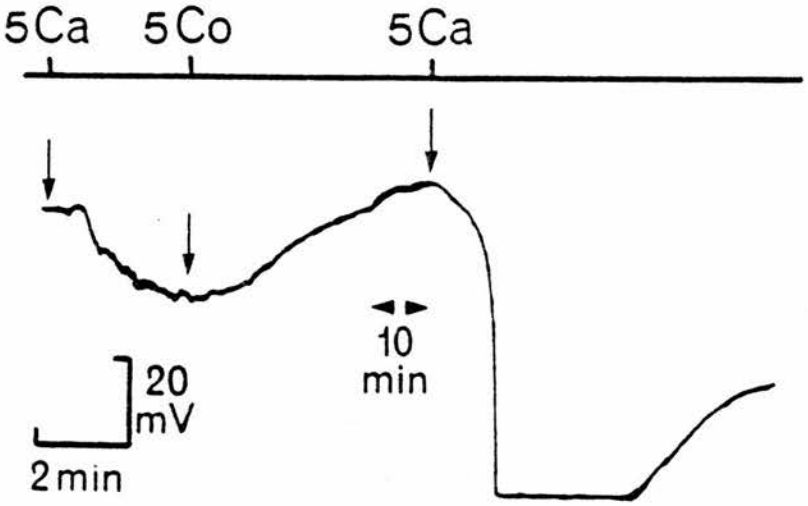



FIGURE 7: Effect on the readmission response of incubation in 'conditioning' solution containing a low concentration of cobalt.

The glands were incubated for 30 min in zero calcium solution containing 0.5 mM cobalt before readmission of 5 mM calcium in control solution (first arrow). After a small readmission response had been elicited the bathing solution was replaced by one without calcium but containing 5 mM cobalt. 12 min later (chart recorder slowed during the cobalt incubation) 5 mM calcium in control solution was reintroduced causing a large hyperpolarization (peak value off-scale here).

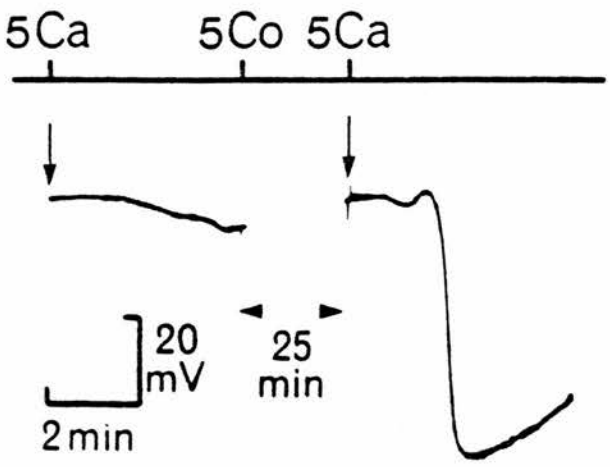



FIGURE 8: Effect on the readmission response of incubation in 'conditioning' solution containing calcium and cobalt.

The glands were incubated in 5 mM cobalt + 5 mM calcium and after 30 min the solution was replaced (first arrow) by control solution containing 5 mM calcium. After a small response was produced the glands were incubated for a further 25 min in 5 mM cobalt medium without calcium. Readmission of control solution then produced a large hyperpolarization (second arrow).

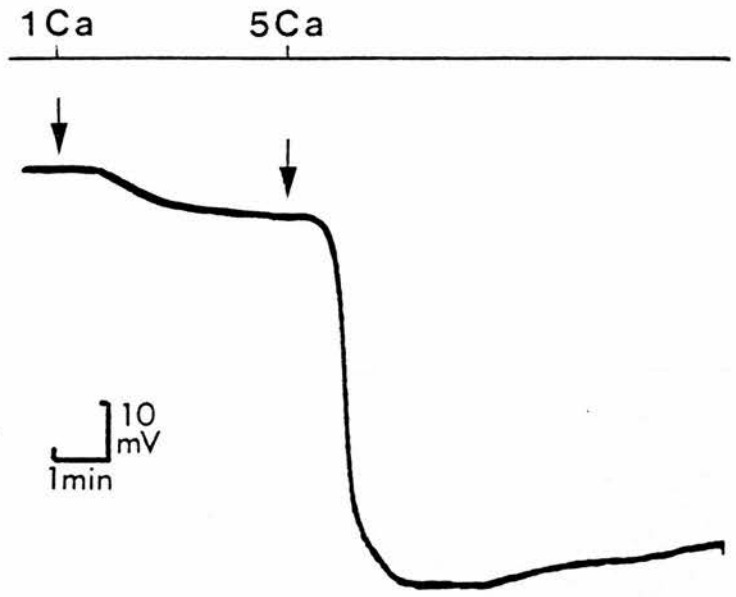



FIGURE 9: Effect of a decreased calcium concentration on the readmission response.

The glands were incubated in calcium-free medium containing 5 mM cobalt for 15 min and then control solution containing 1 mM calcium was introduced (first arrow). A much larger response was elicited when the calcium concentration was increased to 5 mM (second arrow).

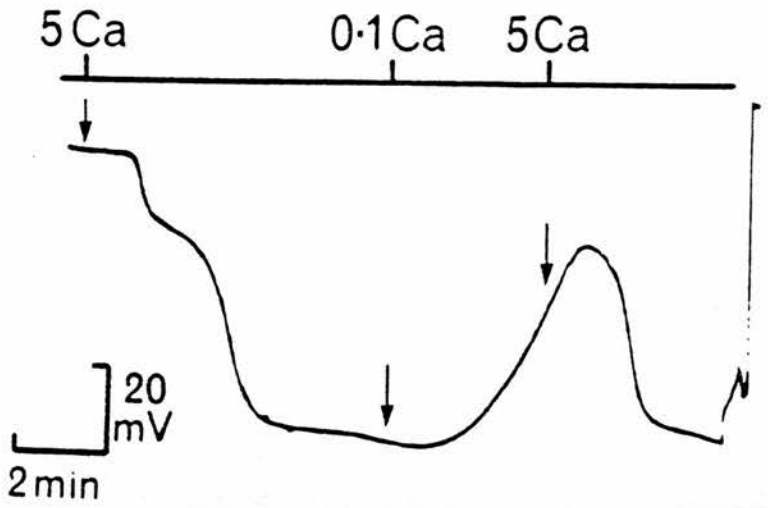



FIGURE 10: Effect of decreasing the calcium concentration after production of the readmission hyperpolarization.

The preparation was incubated in zero calcium solution containing 5 mM cobalt for 30 min before introduction of control solution containing 5 mM calcium (first arrow). Between second and third arrows the calcium concentration was reduced to 0.1 mM. The sharp deflection at the end of the record represents withdrawal of the recording electrode.

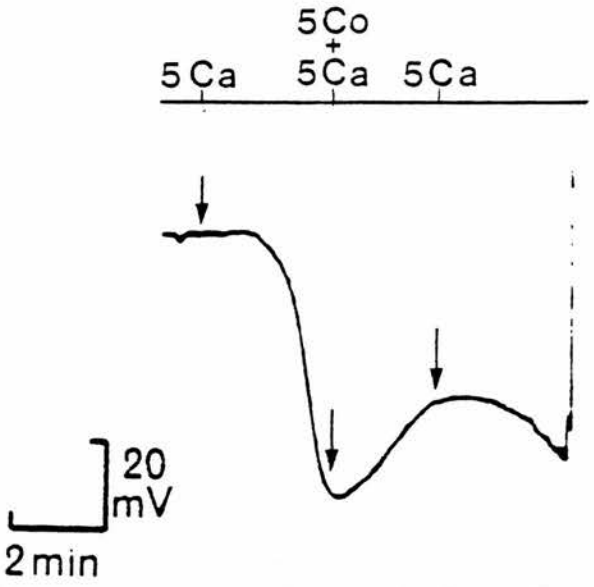



FIGURE 11: Effect of the addition of cobalt to the readmission solution.

Solution containing 5 mM calcium was introduced after 30 min incubation in zero calcium medium containing 5 mM cobalt (first arrow). Between the second and third arrows 5 mM cobalt was added to the control solution.

The sharp deflection at the end of the record signifies removal of the recording electrode.

A prolonged readmission response was also seen after incubation with magnesium-containing calcium-free medium if the readmission fluid contained 5 mM magnesium in addition to 5 mM calcium (Figure 12), and was reversed on return to calcium-free medium. This shows that magnesium has a much weaker effect than cobalt in the enhancement of the calcium readmission response.

Phentolamine and the readmission response

There remained the possibility that the readmission response was produced indirectly through the release of transmitter from the nerve endings and this was investigated with the use of phentolamine, which is known to antagonise the hyperpolarization evoked both by nerve stimulation and by dopamine (Ginsborg et al, 1976; Bowser-Riley et al, 1978).

In 14 experiments phentolamine (0.1 or 0.5 mM) was added to both conditioning and readmission solutions. In five of these no hyperpolarization was elicited within the first 3-minute period after calcium readmission but a response was seen on washout of the phentolamine. In the remaining nine cells responses were evoked within 3 minutes as is typical in preparations not incubated in the presence of phentolamine. Figure 13 shows simultaneous records from two cells, only one of which responded to the readmission of calcium in the presence of phentolamine.

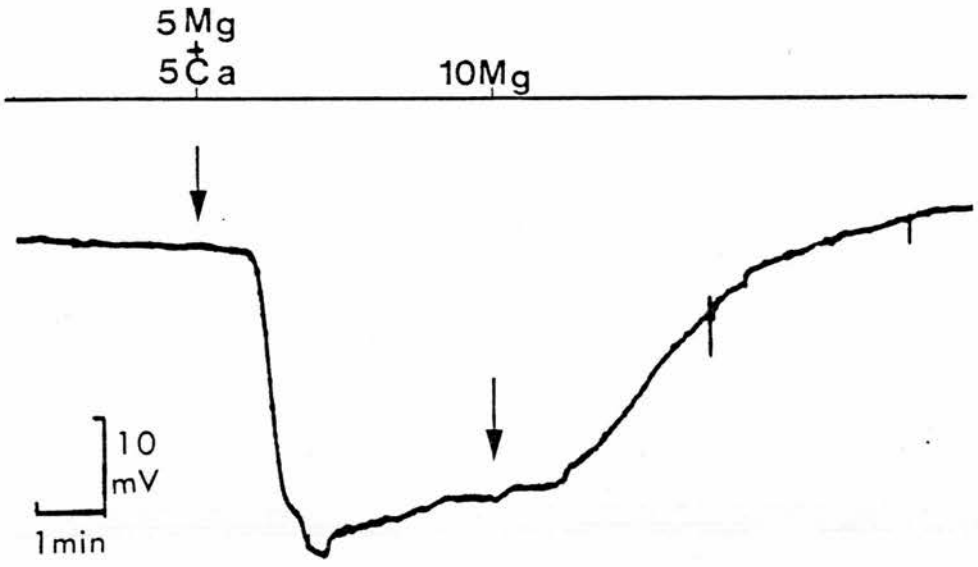



FIGURE 12: Effect of readmission of calcium with magnesium.

The preparation was incubated in zero calcium solution containing 10 mM magnesium for 10 min and then in solution containing 5 mM calcium + 5 mM magnesium (first arrow). The calcium containing solution was replaced at the second arrow by the one containing 10 mM magnesium.

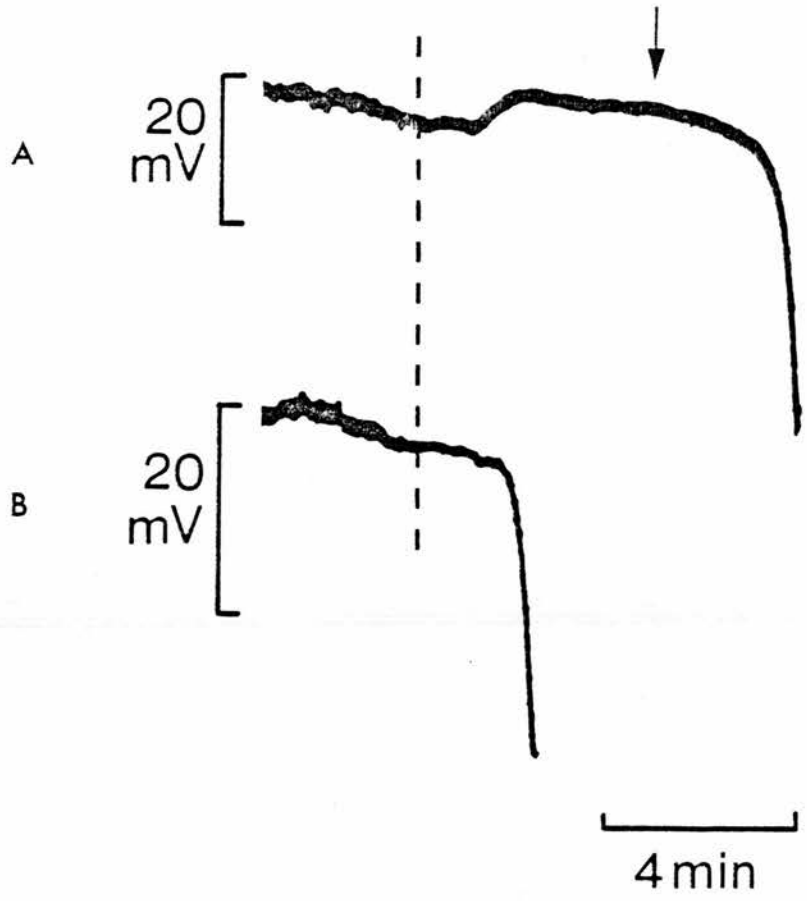



FIGURE 13: Effect of phentolamine on the readmission response.

A and B represent simultaneous recordings from two separate acini. The preparation was incubated for 20 min in calcium-free medium containing 1 mM magnesium, 1 mM EGTA and 5 x $10^{-4}$M phentolamine. Control solution containing 5 mM calcium and 5 x $10^{-4}$M phentolamine was introduced (dotted line). 5 min later (arrow) the glands were perfused with control solution not containing phentolamine.

These results show that phentolamine is able to block the response but does not provide strong evidence for the idea that transmitter release is responsible for the response. A further experiment was performed to determine if the responses elicited in the presence of phentolamine were due to the attainment of a high enough transmitter concentration to overcome the phentolamine blockade. Figure 14A shows that the time course of recovery of the readmission response was unaffected by a prolonged exposure to phentolamine (0.5 mM). This was in marked contrast to the rapid abolition by phentolamine of a prolonged hyperpolarization evoked by dopamine leakage from a micropipette (Figure 14B).

Resistance changes and calcium withdrawal

Douglas (1968) has suggested that calcium deprivation might lead to structural changes in adrenal chromaffin cell membranes resulting in an increase in membrane permeability which would then permit a large calcium influx on readmission of calcium to the bathing medium. In order to investigate this possibility in the salivary glands, the resistance of a cell was measured before and after calcium removal (Figure 15). The top traces show the current passed by one intracellular electrode and the bottom traces show the resulting electronic potentials recorded by a second intracellular electrode in standard (A) and in calcium-free medium containing 1 mM magnesium

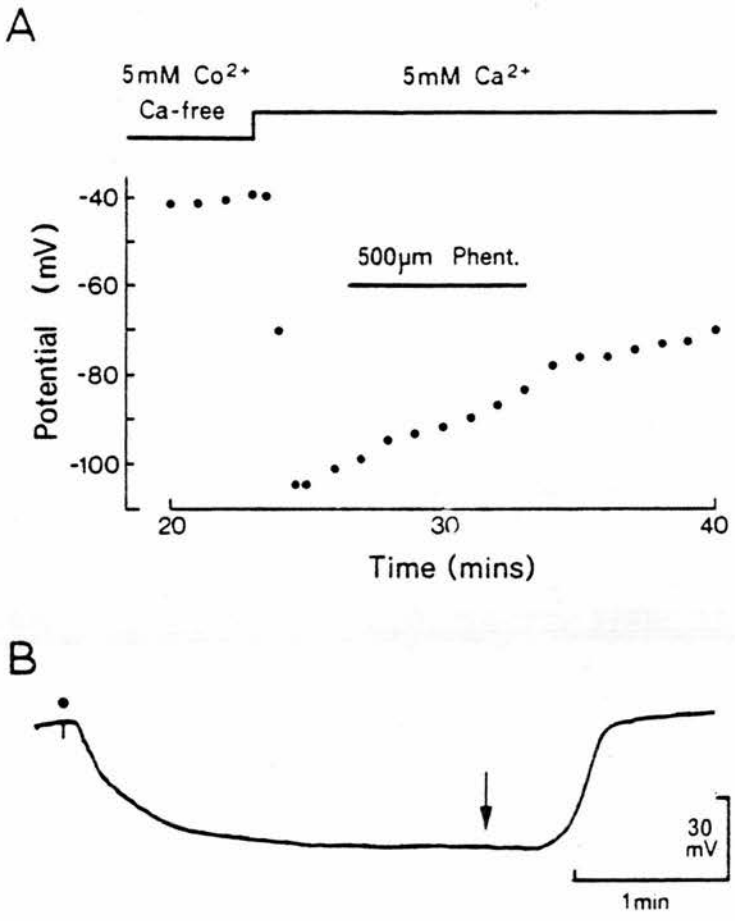



FIGURE 14: Comparison of the effect of phentolamine on the readmission response and the response to dopamine.

A illustrates part of the time course of recovery of the membrane potential of a cell in calcium medium after a 20 min incubation in calcium-free medium containing 5 mM cobalt. 0.5 mM phentolamine was added to the readmission solution during the period shown by the bar.

B shows the rapid effect of 0.5 mM phentolamine on a hyperpolarization evoked by dopamine diffusing from a micropipette. The response to dopamine was still depressed after 20 min in phentolamine-free solution.

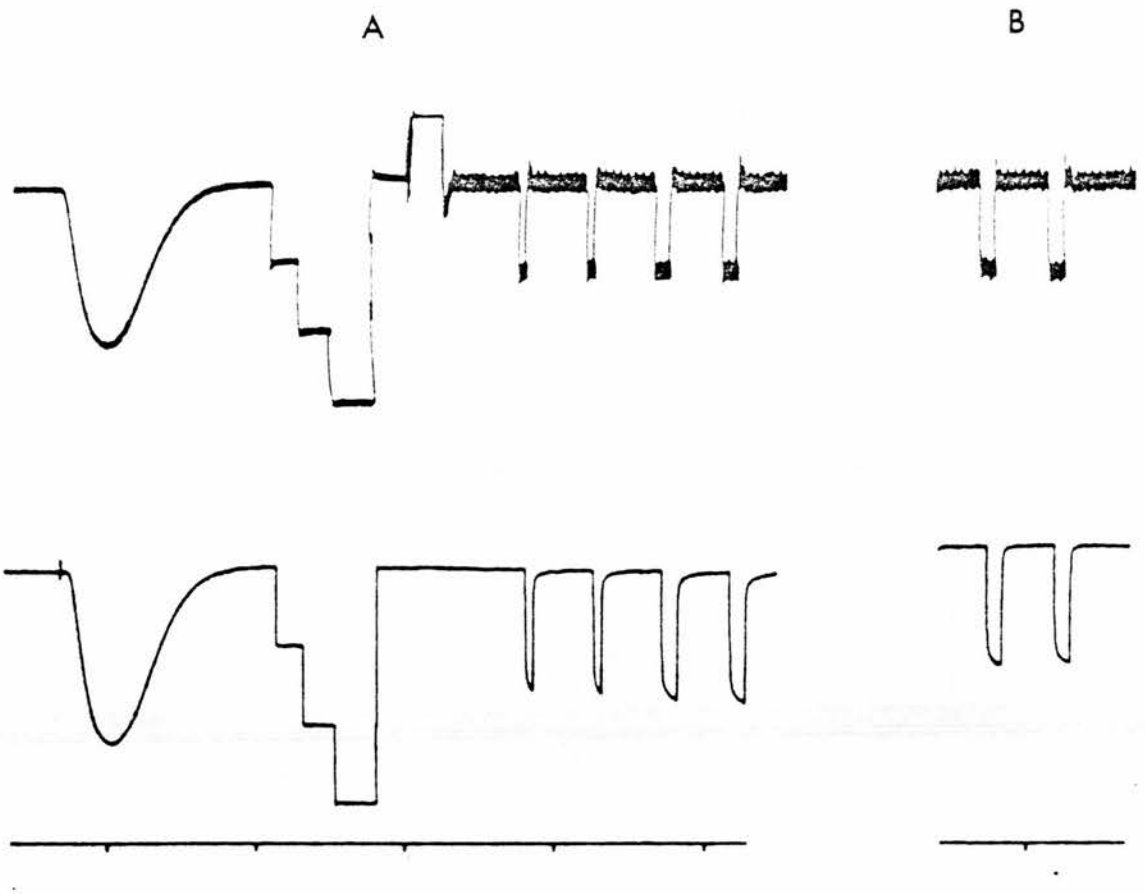



FIGURE 15: Effect on input resistance of withdrawal of calcium from the bathing medium.

Top trace - current pulses applied to one intracellular microelectrode.

Middle trace - electrotonic potentials measured by a second intracellular microelectrode in the same acinus. 10 mV calibrating steps are shown here and in the top trace.

Bottom trace - 10 sec time marks.

A shows responses recorded by both microelectrodes in control solution on stimulation of the salivary nerves.

B shows current pulses and resulting electronic potentials after 17 min in zero calcium soltuion containing 1 mM magnesium + 1 mM EGTA.

+ 1 mM EGTA(B). On changing the calcium-containing bathing medium to the 'zero' calcium solution, there was a depolarization of about 5 mV and a small reduction in input resistance from 0.68 to 0.6 MΩ. In four other experiments calcium removal resulted in varied changes in resistance, there being reductions of 20, 20 and 50% in three experiments and in the fourth an increase of 20%. These results do not support the idea of a generalised increase in conductance of the membrane due to calcium deprivation, but the possibility remains that there is a specific increase in permeability to calcium ions.

Origin of the hyperpolarization:

1. Increase in potassium concentration

Ginsborg, House and Silinsky (1974) have shown that the hyperpolarization due to nerve stimulation or dopamine application is associated with an increase in membrane potassium conductance and it was therefore of interest to investigate whether the same was true for the readmission hyperpolarization. A four-fold increase in the potassium concentration reduced the hyperpolarization by about 25 mV (Figure 16) as compared to 4 mV in resting cells (House, 1973), suggesting that the response is indeed associated with an increase in potassium permeability.

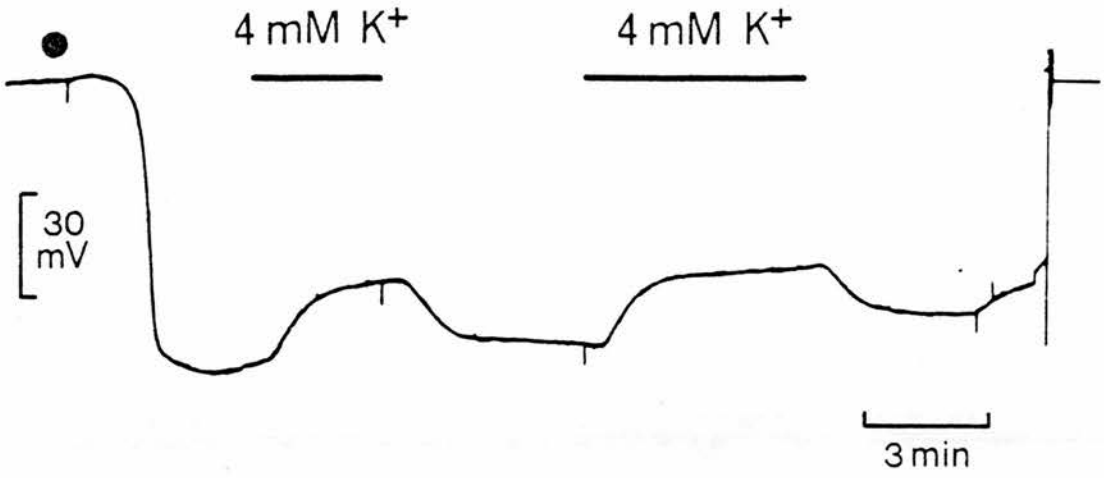



FIGURE 16: Effect of an increased potassium ion concentration on the readmission response.

The preparation was incubated in calcium-free solution containing 5 mM cobalt for 9 min before readmission of control solution containing 5 mM calcium and 1 mM potassium at ●. The potassium concentration was increased to 4 mM over the periods shown by the horizontal bars. The sharp deflection indicates withdrawal of the electrode.

2. Resistance measurements

Figure 17 illustrates the results of an experiment where an acinus was impaled with two electrodes, one for current injection and the other for recording. The conditioning solution contained 5 mM cobalt and the input resistance was found to be 0.5 MΩ. After 30 minutes in this solution, cobalt-free medium containing 5 mM calcium was introduced and a large hyperpolarization (to -117 mV) was elicited together with a decrease in input resistance to about 0.16 MΩ. The changes in membrane potential and resistance were rapidly reversed on return to the calcium-free solution. The decrease in input resistance during the readmission response indicates that an increase in ion permeability occurs during this time.

3. Reversal potential for the readmission response

The above experiments (Figures 16 and 17) suggested that the hyperpolarization was due to an increase in the permeability to potassium ions; if so, the reversal potential for the response would be expected to be equal to the Nernst equilibrium potential for these ions.

The reversal potential was calculated as shown in Methods using data from the experiment illustrated in Figure 18. The bathing solution contained 15 mM instead of 1 mM potassium in order to allow reversal of the response by passing 100 nA current pulses. During the readmission response there was a hyperpolarization to -40 mV with zero current (upper envelope) and a depolariza-

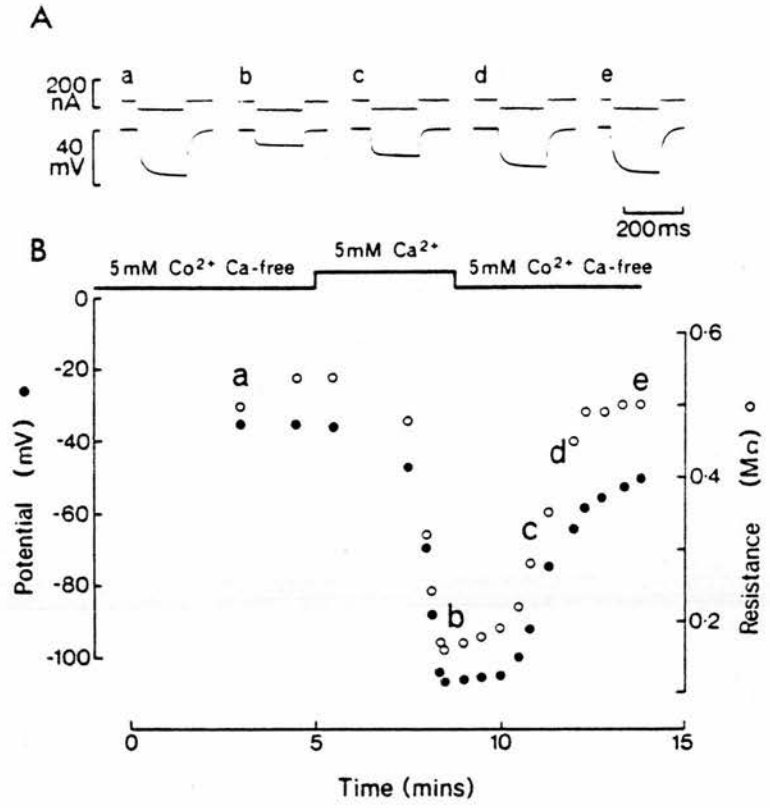



FIGURE 17: Reduction of input resistance associated with the readmission response.

A: upper trace - current pulses through one intracellular electrode;
lower trace - electrotonic potentials recorded with a second intracellular electrode in the same acinus.

B: time course in the changes in resistance (open circles) and membrane potential (filled circles). The letters a - e correspond to the records shown in A.

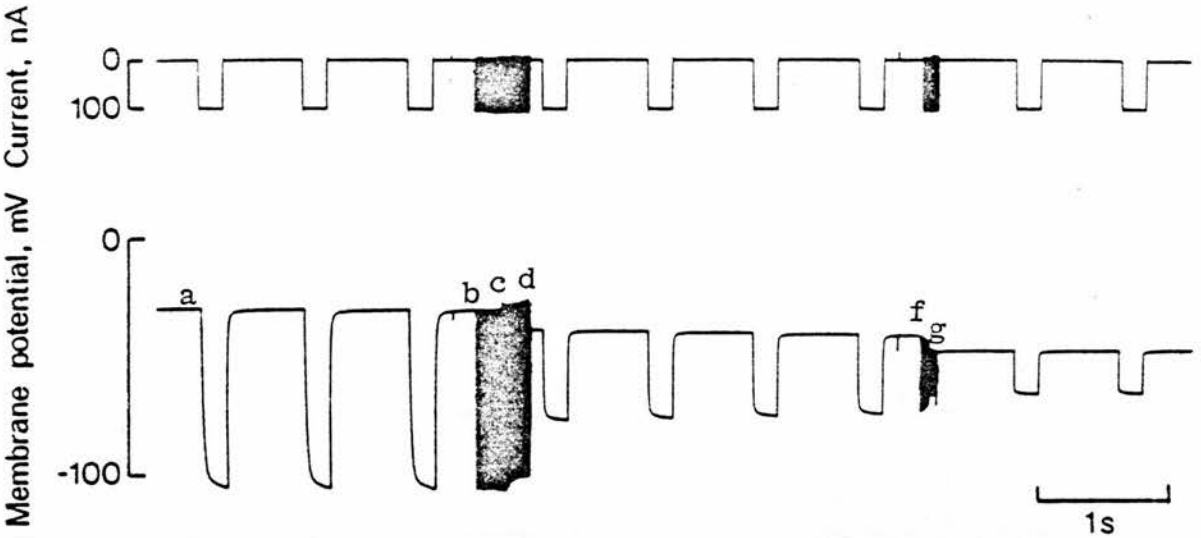



FIGURE 18: Reversal potential for the readmission response.

Upper trace: current pulses through one intracellular electrode.

Lower trace: electrotonic potentials recorded by a second intracellular electrode in the same acinus.

The recording speed was reduced by a factor of 60 at b, a further factor of 50 at c, restored at d, slowed by a factor of 60 at f and restored at g. At a, the preparation was in calcium-free medium containing 5 mM cobalt and at b, control solution containing 5 mM calcium was introduced. Both solutions contained 15 mM potassium. The reversal potential lies between the lower and upper 'envelopes' of the electrotonic potentials in the control solution.

tion to -60 mV with 100 nA current (lower envelope). The reversal potential therefore lies between these two values and was calculated to be -51 mV. From the Nernst equation, the internal potassium ion concentration can be estimated as 110 mM which implies that the reversal potential in 1 mM external potassium should be -118 mV, a value similar to that approached at the peak of the response in 1 mM potassium (c.f. Figure 17).

The large hyperpolarization seen on calcium readmission may therefore be plausibly attributed to an increase in potassium conductance. A more detailed calculation may be made as follows, with reference to Fibure 1B. Let E = -40 mV be taken for the resting potential and R = 0.5 MΩ for the input resistance. If it is assumed that the response in 1 mM potassium is due to opening of potassium channels corresponding to a reversal potential of -118 mV (e) and resistance of 0.2 MΩ (r), then the peak hyperpolarization may be calculated as -60 mV which is in reasonable agreement with observed values (Table 1).

Effect of dopamine during the readmission response

In a few experiments dopamine was applied iono-phoretically during the prolonged hyperpolarization and a depolarization was occasionally seen (Figure 19A). The depolarization was similar to that seen in response to nerve stimulation and ionophoretic dopamine in a cell with an unusually high resting potential of -90 mV (Figure 19B). It has already been reported (House, 1973; Ginsborg and House, 1976) that in normal conditions

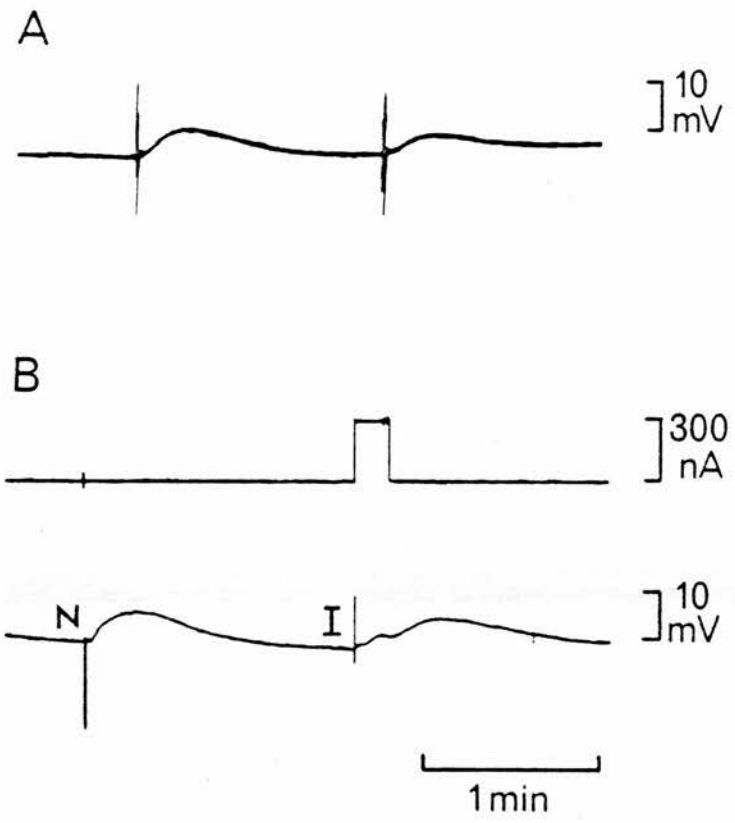



FIGURE 19: Depolarizing responses to ionophoretic dopamine and nerve stimulation.

A: responses to two consecutive short pulses of dopamine at the peak of the readmission response (membrane potential, -100 mV).

B: lower trace shows responses to nerve stimulation (N) and to a pulse of dopamine (I, upper trace). The cell had an unusually high resting potential of -90 mV compared with other cells whose resting potentials were in the normal range. The preparation was in normal solution, but had previously been exposed to one with no added divalent cations for about 1 hour.

dopamine or nerve stimulation can sometimes evoke a hyperpolarizing response with a later, slow depolarizing phase. Since the depolarization to dopamine is seen at the height of the readmission response it represents an underlying membrane potential change which is presumably not evoked by calcium readmission.

## DISCUSSION

### Zero calcium and calcium readmission

The readmission hyperpolarization is due to a direct effect on acinar cells rather than to an indirect effect involving the prolonged release of transmitter because phentolamine ($5 \times 10^{-4}$M) did not reverse the sustained hyperpolarization after incubation in cobalt medium whereas it caused a rapid blockade of a receptor-mediated hyperpolarization (Figure 14). However, phentolamine was sometimes seen to inhibit production of the hyperpolarization. The reason for this is speculative but one possibility is that the calcium influx sites are linked to the dopamine receptor and phentolamine is capable of obstructing access of calcium to these sites.

Removal of calcium from the bathing fluid sometimes caused a depolarization while the changes in input resistance were found to be of variable magnitude and direction. Although other divalent ions may substitute for calcium in stabilizing excitable membranes (Frankenhaeuser and Hodgkin, 1957; Bülbring and Tomita,

1970; Krnjević, Lamour, MacDonald and Nistri, 1979a), the large response seen on calcium readmission after incubation in magnesium or cobalt-containing solution suggests that calcium deprivation produced an increase in permeability specific to calcium. The measurements of resistance also do not support the idea of a generalised increase in permeability in calcium deficient solution (Douglas, 1968).

It should be noted that in later experiments (Section I, Part 3) higher resting potentials (mean = -54 mV) were recorded using improved experimental techniques than those (mean = -32 mV; House, 1973) recorded from the glands using similar apparatus to that employed in the present experiments. On incubation in calcium-free media smaller membrane potentials were recorded (mean = -36 mV). In order to investigate the cause of the depolarization in calcium-free media it would be necessary to monitor input resistance on changing to the zero calcium solution.

Removal of calcium has been found to depolarize parotid gland cells (Petersen and Pedersen, 1974), pancreatic acinar cells (Petersen and Ueda, 1976), Helix central neurones (Moreton, 1972) and smooth muscle of the guinea-pig taenia coli (Brading, Bülbring and Tomita, 1969). Petersen and Ueda and Brading et al suggested that the decrease in resistance associated with the depolarization in calcium-free solution was due to an increase in permeability to sodium ions, but the

depolarization seen in Helix neurones was found to be independent of external sodium. Depolarization could also arise from a decrease in the permeability to potassium and in this case an increase in resistance would be found in calcium-free medium. Indeed, Bülbring and Tomita (1969) found an increase in input resistance associated with the depolarization of smooth muscle in solutions containing a low concentration of calcium suggesting that a decrease in potassium permeability also occurs in smooth muscle in calcium deficient medium.

Readmission of calcium to the above preparations results in hyperpolarization to the initial resting potential, in contrast to the large increase in internal negativity seen on readmission of calcium to cockroach salivary gland acinar cells.

There is no perceptible increase in fluid secretion in the cockroach salivary gland on calcium readmission after incubation in test solution without divalent cations or with 5 mM magnesium, but a prolonged, sub-maximal increase in secretion is seen in calcium medium after incubation in 5 mM cobalt (C.R. House, personal communication). Readmission of calcium in pancreatic acinar cells evokes a small, transient increase in amylase output (Petersen and Ueda, 1976) and in the submaxillary gland a small increase in secretion is occasionally seen (Douglas and Poisner, 1963). This contrasts with the large increase in secretion on calcium readmission in the adrenal gland (Douglas and

Rubin, 1961). In the endocrine pancreas a large increase in secretion is also evoked by readmission of a high (18 mM) concentration of calcium and, on reaching a maximum insulin output some 10 - 16 minutes after calcium readmission, insulin output declines to the basal release rate (Devis, Somers and Malaisse, 1977). The prolonged increase in secretion in the cockroach salivary gland after cobalt treatment occurs concomitantly with the large hyperpolarization on calcium readmission suggesting a connection between the two events. An increase in secretion after incubation in calcium-free medium without cobalt may not be detected due to the transient nature of the readmission response or to differences in the calcium requirement for secretion and for hyperpolarization.

Calcium-dependent potassium activation and effect of cobalt

The hyperpolarization evoked on readmission of calcium to cockroach salivary glands is due to an increase in potassium permeability. Meech and Strumwasser (1970), using the mollusc Aplysia, originally proposed that intracellular calcium ions could activate potassium channels in nervous tissue, and similar findings have been made in Helix aspersa neurones (Meech, 1974a). Intracellular injection of calcium ions into mammalian spinal neurones also increases the potassium conductance (Krnjević and Lisiewicz, 1972). The calcium dependent potassium activation in these neurones underlies the post-

spike afterhyperpolarization as is the case in the guinea-pig myenteric plexus (Nishi and North, 1973; Hirst, Holman and Spence, 1974) and in sympathetic (Busis and Weight, 1976) and parasympathetic (Suzuki and Kusano, 1978) ganglion cells. Intracellular calcium also regulates the potassium conductance in cardiac Purkinje fibres (Isenberg, 1977a,b; Siegelbaum and Tsien, 1979) and in ventricular muscle (Bassingthwaighte, Fry and McGuigan, 1976) and thus has control over the duration of the action potential.

This type of potassium activation may be antagonised by intracellular injection of EGTA (Krnjević, Puil and Werman, 1975, 1978; Meech, 1974a,b; Isenberg, 1977a) or by the divalent cations, cobalt and magnesium (Barrett and Barrett, 1976; Krnjević et al, 1979b; Moolenaar and Spector, 1979), and may be potentiated by any agent which increases intracellular calcium, e.g. caffeine (Suzuki and Kusano, 1978; Morita, Koketsue and Kuba, 1980).

Further evidence that there is an increase in intracellular calcium ion concentration comes from the use of the dyes, arsenazo III (Thomas and Gorman, 1977) and aequorin (Eckert and Tillotson, 1978) and shows that the change in membrane potential is closely associated with the change in absorbance due to the increase in intracellular calcium.

In the experiments reported here it seems that the increase in potassium permeability arises from an increase

in intracellular calcium due to influx from the external solution. Cobalt appears to prolong this influx rather than to inhibit calcium binding in intracellular stores because the readmission response after cobalt treatment is only sustained if a high calcium concentration is present externally. The magnitude of the readmission response is markedly reduced if the concentration of cobalt in the conditioning solution is low or if calcium is present with cobalt suggesting that the large increase in permeability is dependent on incubation in a high cobalt concentration and that there may be competition by calcium for the sites at which cobalt acts. Further evidence for competition between cobalt and calcium comes from the observation that the readmission response was reduced by introduction of a solution containing calcium and cobalt. The competition between the two ions may occur at two different calcium binding sites, (a) concerned with regulation of calcium permeability, and (b) concerned with calcium influx. Site (b) seems to be a more superficial site and removal of cobalt from solution allows calcium to act at these sites (Figure 11). Site (a), however, would retain cobalt after washout of the cobalt-containing solution and the cobalt action at this site may result in prolongation of a conformational change, arising from calcium depletion, which would allow a sustained influx of calcium via the second calcium binding site.

The enhancement of the calcium permeability was also seen after magnesium incubation but only if magnesium was present in the readmission fluid with calcium. The much more potent effect of cobalt in prolonging the increase in permeability to calcium may be due to it having a greater affinity than magnesium for the calcium binding sites (a) and this idea is supported by the observation that cobalt is twenty times more potent an inhibitor of calcium-dependent transmitter release than is magnesium (Weakly, 1973).

The hyperpolarization evoked by calcium readmission is similar to that evoked by nerve stimulation and by the putative transmitter dopamine in that it has a comparable reversal potential, reflecting an increase in potassium permeability (Ginsborg et al, 1974). It is therefore reasonable to suppose that the hyperpolarization observed on nervous stimulation or dopamine application also results from an initial increase in the cytosolic calcium concentration and this is investigated further in the following experiments.

# SECTION I: PART 2

## The role of calcium in the hyperpolarization of salivary gland acinar cells

## Introduction

In the preceding section it was proposed that a hyperpolarization of the acinar cell could be evoked by an increase in potassium permeability activated by calcium ions following their influx from the external medium. Hyperpolarizing responses resulting from nerve stimulation or dopamine application in the cockroach salivary gland have also been associated with an increase in potassium permeability (House, 1973; Ginsborg et al, 1974) and it was therefore of interest to determine the importance of calcium in these evoked responses. The results suggest that there may be a buffered store of calcium in the acinus which can be replenished from the external solution and that stimulation by dopamine results in influx of calcium from the store to mediate activation of potassium channels.

## METHODS

The control solution was as described in General Methods and the test solutions (see Table 1) contained varying concentrations of calcium (0.01 to 0.5 mM). Zero calcium solutions were without divalent cations or contained magnesium or cobalt. From the maximum calcium impurity specified for the reagents used in the calcium-free solutions it is likely that the calcium content is less than $10^{-7}$M. In a few experiments, EGTA was also added thus reducing any free calcium ion concentration still further, probably to less than $10^{-9}$M (Hubbard, Jones and Landau, 1968).

RESULTS

Low calcium solutions

Reduction of the external calcium ion concentration from 5 mM to 0.5 mM diminished the hyperpolarizing responses to nerve stimulation while the magnitude of the responses evoked by ionophoretic dopamine remained the same (Figure 1); this shows that transmitter release is highly dependent on the external calcium ion concentration. The ionophoretic responses were maintained even after prolonged exposure to even lower calcium concentrations, e.g. 300 μM, but a reduction was seen with very low concentrations of calcium, e.g. 20 μM (Table 1).

Zero calcium solution

On exposure of the salivary glands to calcium-free medium, the resting potential often decreased although, occasionally, there was no change or even an increase was observed (Table 1). There was also no consistent change in resistance on removal of calcium (c.f. Section I, Part 1).

Responses to ionophoretically applied dopamine could still be evoked even after a prolonged exposure to calcium-free solution (Figure 2). On return to control solution containing 5 mM calcium a readmission hyperpolarization was elicited (c.f. Part 1) and the response to a pulse of dopamine was restored to the same magnitude as that seen before exposure to the calcium-free medium (Figure 3).

Although the amplitude of the hyperpolarization to a particular dopamine charge is reduced in calcium-free

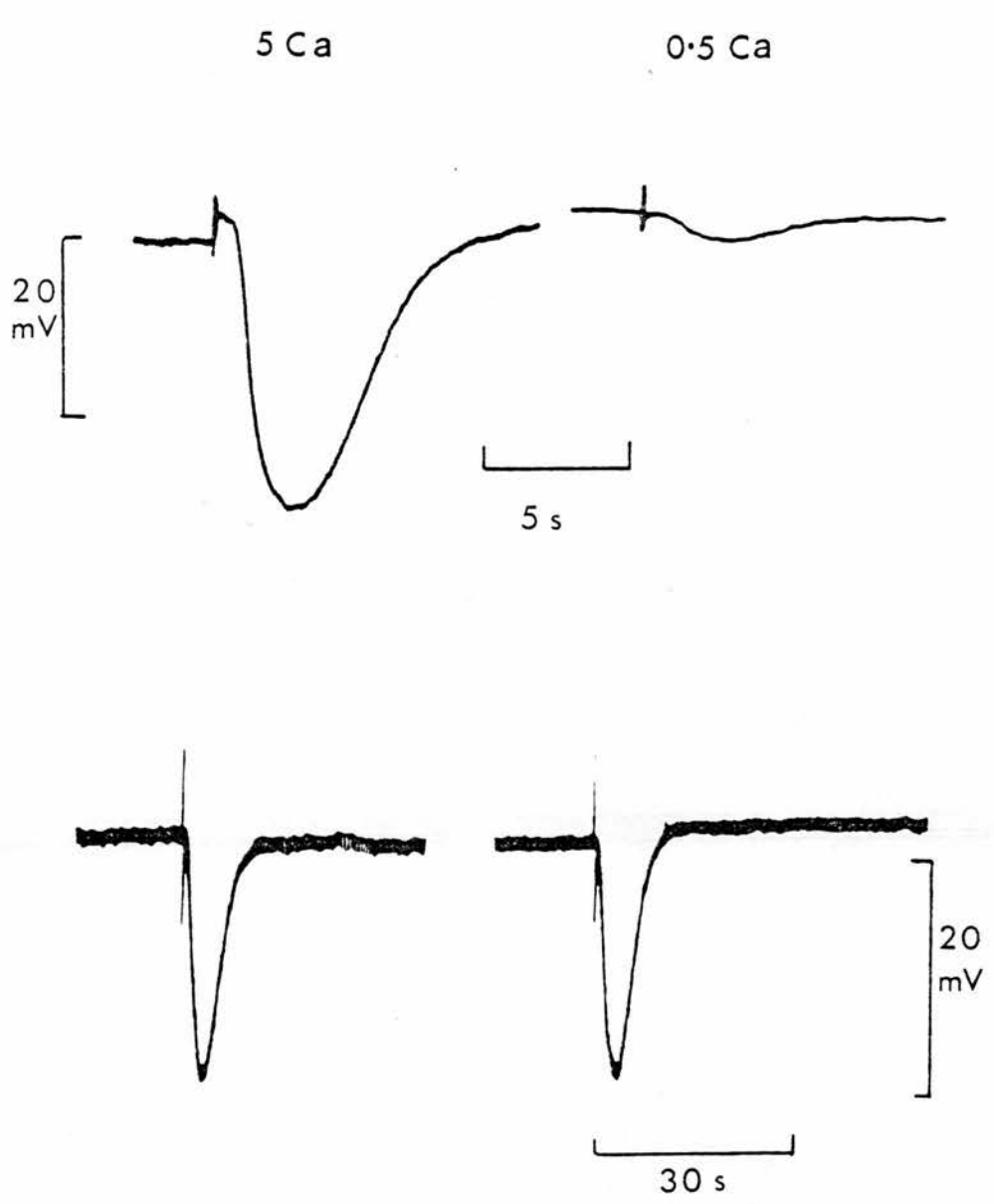



FIGURE 1: Effect of reduction of concentration of calcium in the bathing fluid on responses to nerve stimulation and ionophoretic dopamine.

Upper traces: responses to a burst of 10 stimuli applied to the salivary nerve.

Lower traces: responses to dopamine ejected by brief ionophoretic current pulses (20 nA, 200 ms).

The first of each pair of responses was recorded in control solution containing 5 mM calcium. The second of each pair was recorded in solution containing 0.5 mM calcium.

TABLE 1: Effect of changing from control solution containing 5 (or 10*) mM $Ca^{2+}$ to test solution on the amplitude of the response of acinar cells to dopamine applied ionophoretically by a brief current pulse delivered to a micropipette containing 0.1 to 0.5 M dopamine in 3M K-acetate.

| Test solution contains (mM) | Control amplitude (mV) | Test amplitude (mV) | Time in test solution (min) | Change to resting potential[1] (mV) |
|---|---|---|---|---|
| 0.5 Ca | 17 | 17 | 12 | 0 |
| 0.3 Ca | 8 | 7 | 10 | 0 |
| 0.1 Ca | 7 | 8 | 35 | 0 |
| 0.02 Ca | 20 | 8 | 12 | -10 |
| no added divalent ions | 16 | 0 | 12 | -5 |
| | 40 | 23 | 20 | +10 |
| | 30 | 0 | 7 | -5 |
| | 30 | 2.5 | 14 | -7 |
| | 26 | 10 | 7 | 0 |
| | 23 | 5 | 11 | +3 |
| no added divalent ions + 1 EGTA | 30 | 0 | 6 | +20 |
| | 25 | 0 | 4 | +20 |
| | 20 | 0 | 6 | +20 |
| | 19 | 0.5 | 5 | +20 |
| 1 Mg + 1 EGTA | 60 | 30 | 10 | +5 |
| | 55 | 5 | 6 | +5 |
| | 25 | 0 | 8 | +6 |
| | 51 | 5 | 12 | +6 |
| 10 Mg | *45 | 7 | 13 | +10 |
| | 27 | 3 | 5 | +10 |
| | 20 | 5 | 6 | +8 |
| | *20 | 5 | 5 | 0 |
| | 10 | 4 | 6 | 0 |
| | 10 | 3 | 8 | +10 |
| 2.5 Co | 50 | 8 | 16 | +10 |
| | 40 | 0 | 20 | 0 |
| 5 Co | 48 | 1.5 | 30 | +8 |
| | 34 | 0 | 10 | +15 |
| | 34 | 3 | 23 | +5 |
| | 25 | 7 | 12 | +3 |

[1]hyperpolarization given as negative

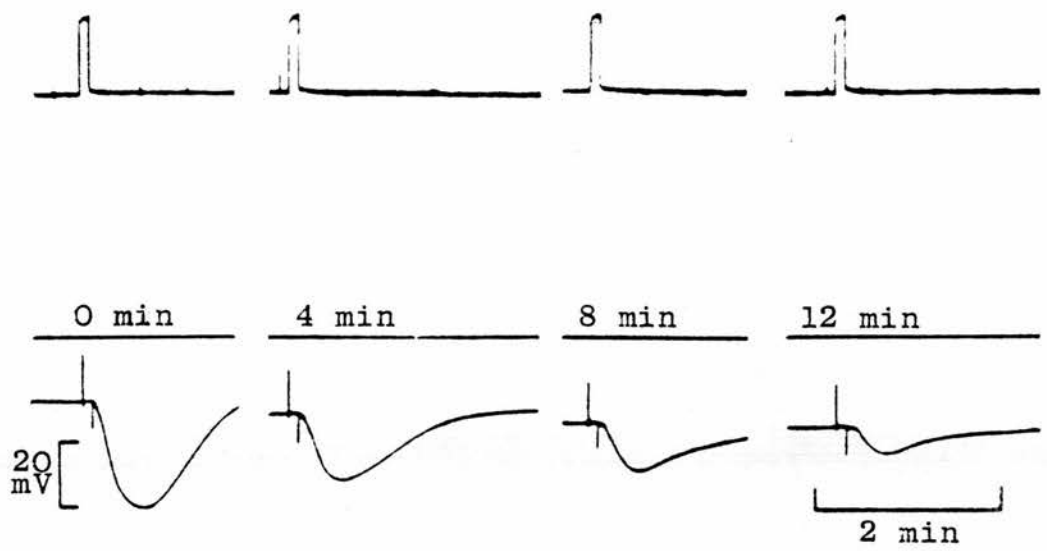



FIGURE 2: Effect of withdrawal of calcium from the bathing solution.

The control solution contained 5 mM calcium; the test solution contained no added calcium. Responses (bottom traces) at indicated times after introduction of test solution are to dopamine ejected by 120 nA ionophoretic current pulses (top traces). Middle traces correspond to a level of -20 mV. The cell hyperpolarized by about 9 mV in the test solution.

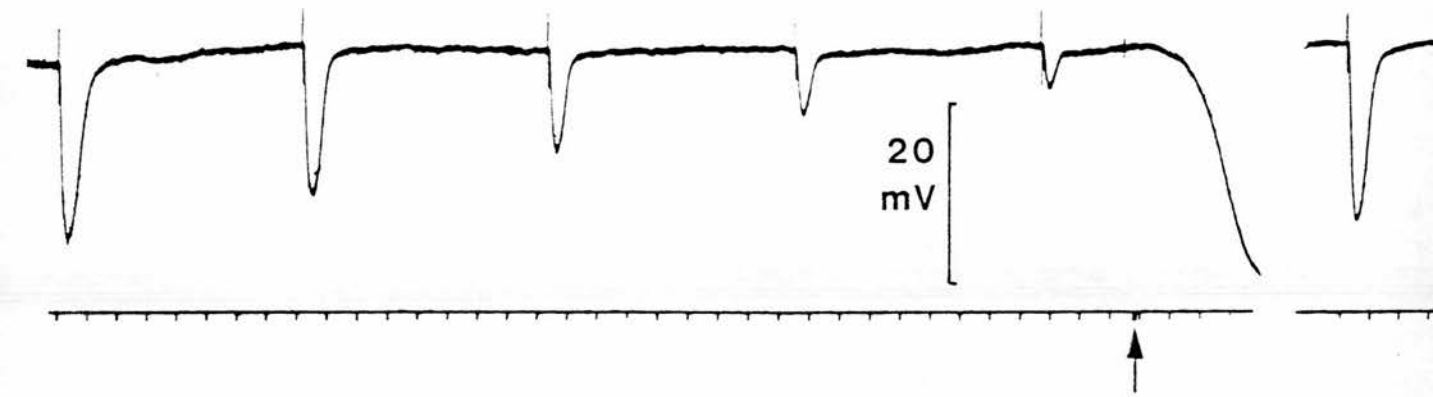



FIGURE 3: Effect of replacing calcium in the bathing fluid by magnesium.

The control solution contained 10 mM calcium; the test solution contained 10 mM magnesium. Lower trace shows 10 sec time marks and signal indicating return of control solution (arrow). Test solution introduced immediately before recording shown. Upper trace shows response to dopamine ejected by brief 10 nC ionophoretic pulses. Note 'spontaneous' hyperpolarization when control solution was readmitted. The final response was obtained in control solution 4 min later when the resting potential was at its control level.

solution, the original amplitude may be restored by increasing the amount of dopamine ejected (Figure 4, 5). Figure 4 illustrates the effects of calcium withdrawal on responses to two different quantities of dopamine, applied alternately. The smaller response is rapidly abolished in zero calcium while the larger response is only reduced by about half in the same period. It is not likely that the reduction in the hyperpolarizations is due to a general reduction in resistance in zero calcium because no consistent, marked reduction in input resistance was found in calcium-free medium (see Part 1).

Zero calcium solutions containing EGTA

In order to be more certain that the calcium concentration in the vicinity of the acinar cells was very low and that the above responses in 'zero' calcium were not due to residual calcium in the solutions, six experiments were carried out in calcium-free solution containing 1 mM EGTA. Figure 5 illustrates one such experiment and shows a reduction in the response to a small pulse of dopamine in 1 mM $Mg^{2+}$ + 1 mM EGTA (Figure 5A2), which could be surmounted by application of a large dopamine pulse (Figure 5A3). A transient hyperpolarization was seen on readmission of control solution and the responses to applied dopamine quickly recovered their original magnitude.

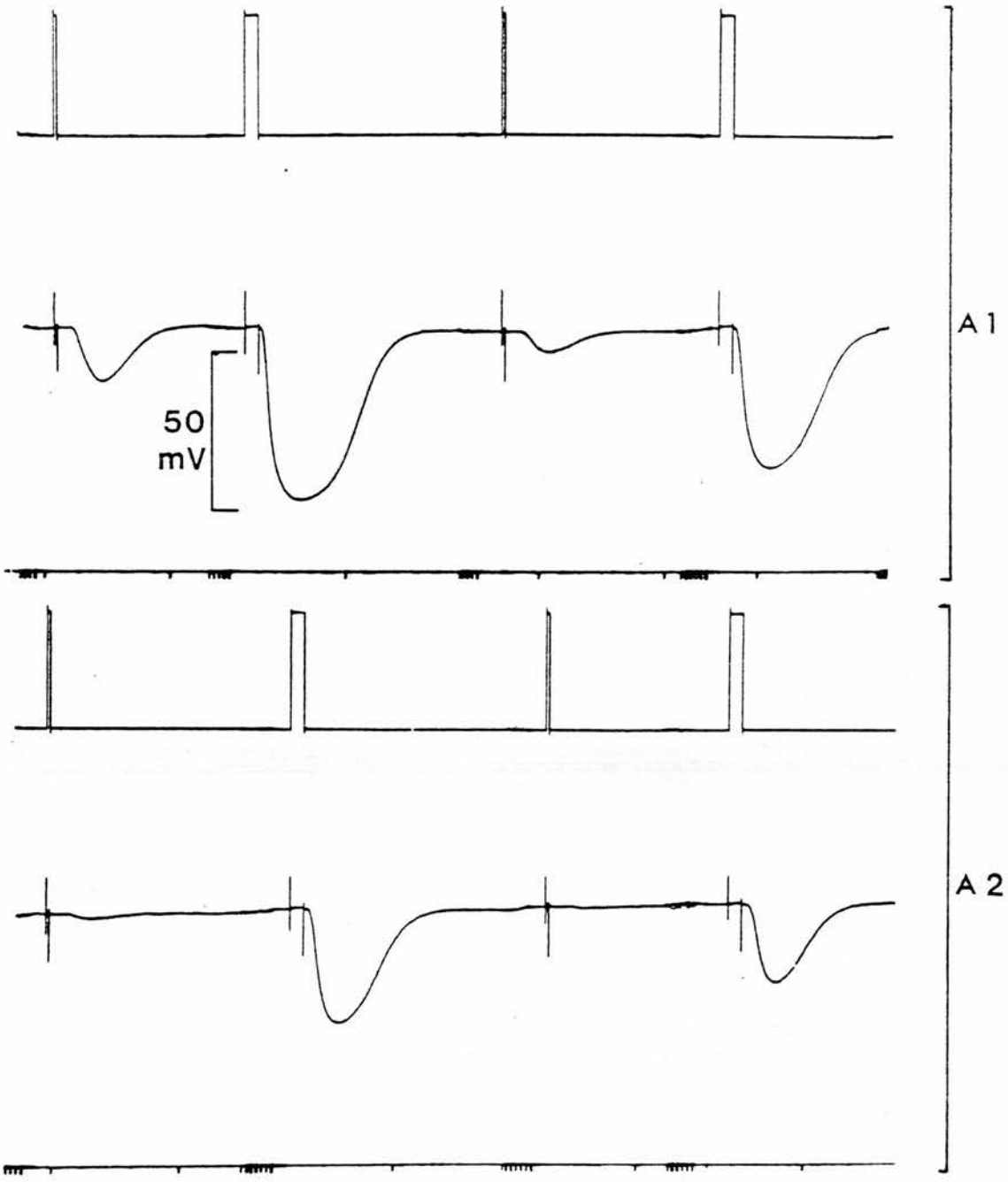



FIGURE 4: Effect of withdrawal of calcium from the bathing solution.

The control solution contained 5 mM calcium; the test solution contained no added calcium. Segments of a continuous recording are shown in A1 and A2. Bottom traces show 10 sec time marks; chart speed reduced between stimuli. Responses (middle traces) to dopamine ejected by 1 μA ionophoretic pulses (upper traces) alternately of 0.25 and 1 s in test solution applied 3 min before beginning of A1. There was a progressive depolarization of 7 mV during A1 and A2.

FIGURE 5: Effect of withdrawal of calcium from the bathing solution.

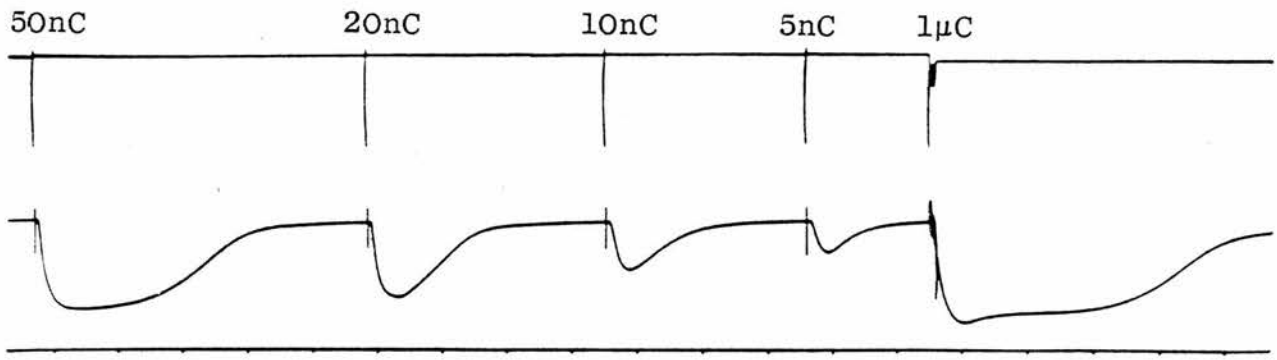
The control solution contained 5 mM calcium; the test solution contained 1 mM magnesium + 1 mM EGTA. Segments of a continuous recording are shown in A1, A2 and A3; between the end of A1 and beginning of A2 there was an interval of 2 min and the corresponding interval between A2 and A3 was 9 min. Bottom traces show 10 s time marks (and in A3 also 1 min intervals when chart speed was reduced). Small arrows indicate 10 mV calibration steps.

A1: responses (middle trace) to dopamine ejected by the indicated ionophoretic charges (upper trace) in control solution which was changed at the end of A1.

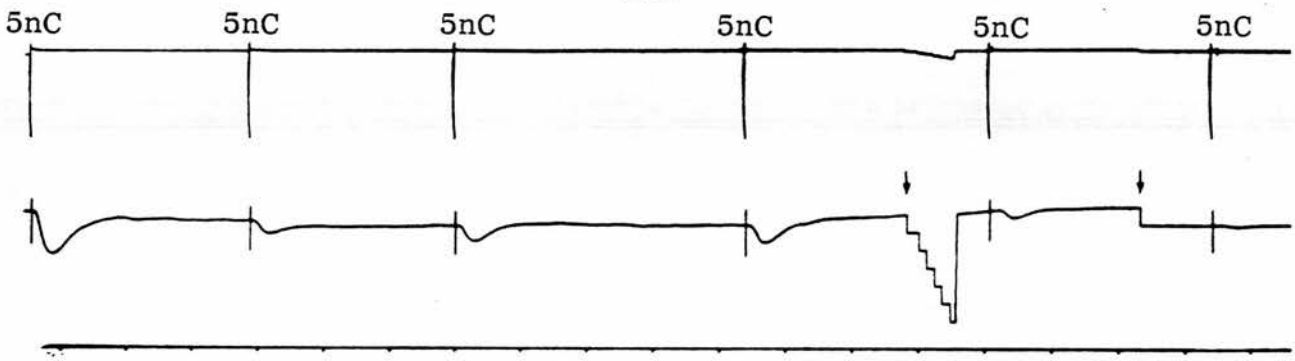
A2: responses in test solution.

A3: test solution present until small arrow. When control solution was readmitted, a large 'spontaneous' hyperpolarization occurred (large arrow).

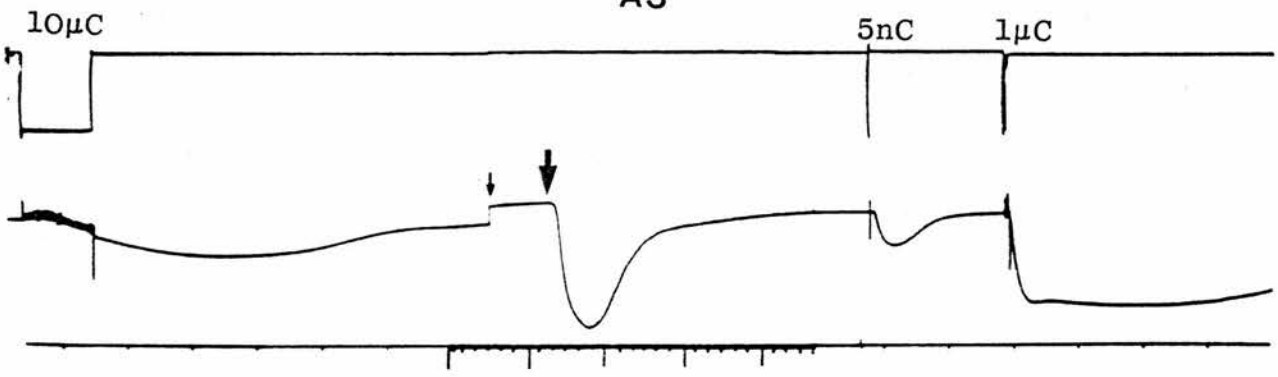
A1




A2




A3



Responses evoked by prolonged pulses of dopamine

The ability of dopamine to evoke responses after prolonged exposure of the salivary gland cells to solutions whose calcium ion concentration is likely to be extremely small suggests that these responses are not dependent on influx of calcium from the external medium. Assuming that the responses are activated by an increase in intracellular calcium ion concentration (c.f. Part 1), dopamine must release calcium from a buffered store in the acinus, which is unaffected by removal of calcium from the external fluid, or by the presence of EGTA.

Figure 6 shows responses to repeated, prolonged dopamine pulses in control solution (A1), in calcium-free medium (A2) and again in control solution (A3). In control solution, both before and after calcium deprivation, repeated doses of dopamine gave responses of the same magnitude. However, in the calcium-free medium, successive responses to the same dopamine charge are reduced in amplitude (Figure 6A2). A calcium readmission response was seen on return to control medium and the fact that successive responses were not reduced in this solution shows that the reduction in the calcium deficient medium was not due to a concomitant deterioration of the cell and loss of ability to produce a marked hyperpolarization. Figure 7 shows a similar reduction in a preparation which had been exposed to zero calcium solution for 80 minutes. The progressive decrease is seen as a result of repetitive stimulation,

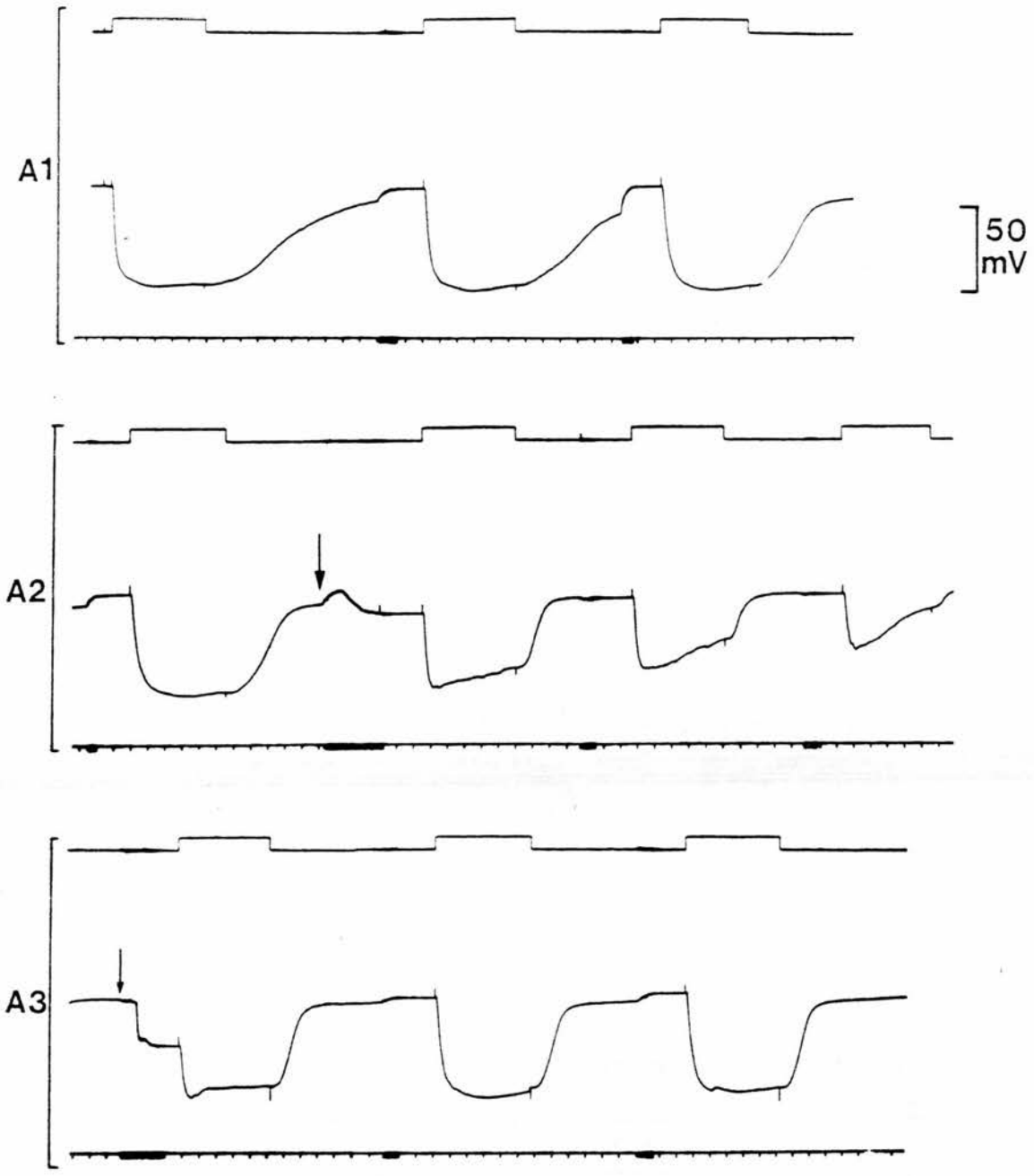



FIGURE 6: Effect of withdrawal of calcium from the bathing solution.

The control solution contained 5 mM calcium; the test solution contained no added calcium. Segments of a continuous recording are shown in A1, A2 and A3. Bottom traces show 10 s time marks: where these cannot be resolved the chart speed was reduced 25-fold. A1: responses (middle trace) to dopamine ejected by ionophoretic current pulses (100 nA) shown in top trace. A2: test solution introduced at arrow. A3: control solution readmitted at arrow; note 'spontaneous' hyperpolarization which followed.

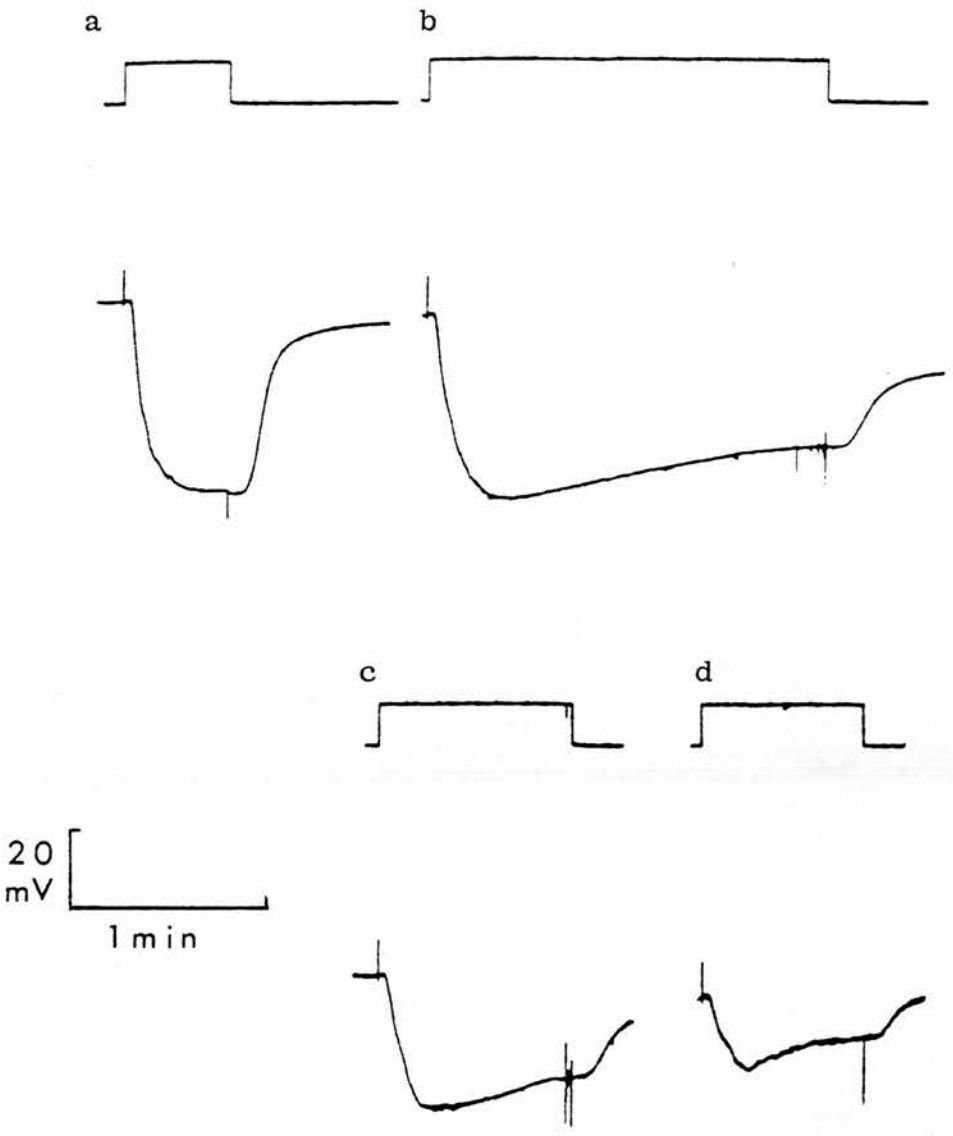



FIGURE 7: Effect of repeated dopamine stimulation after prolonged exposure to calcium-free solution.

The upper traces show 200 nA pulses applied to the dopamine pipette and the lower traces show the subsequent responses. The first response was elicited after the preparation had been left (unstimulated) in bathing solution with no added calcium for 80 min. The following responses were evoked at 83, 93 and 103 min.

suggesting that dopamine gradually exhausts a store of calcium in the acinus. The run-down of responses is not seen in control solution or in solution containing a calcium concentration as low as 50 μM (Figure 8) which suggests that the store is capable of being replenished from the outside medium, even at very low calcium concentrations.

'Zero' calcium solutions containing high concentrations of magnesium or cobalt

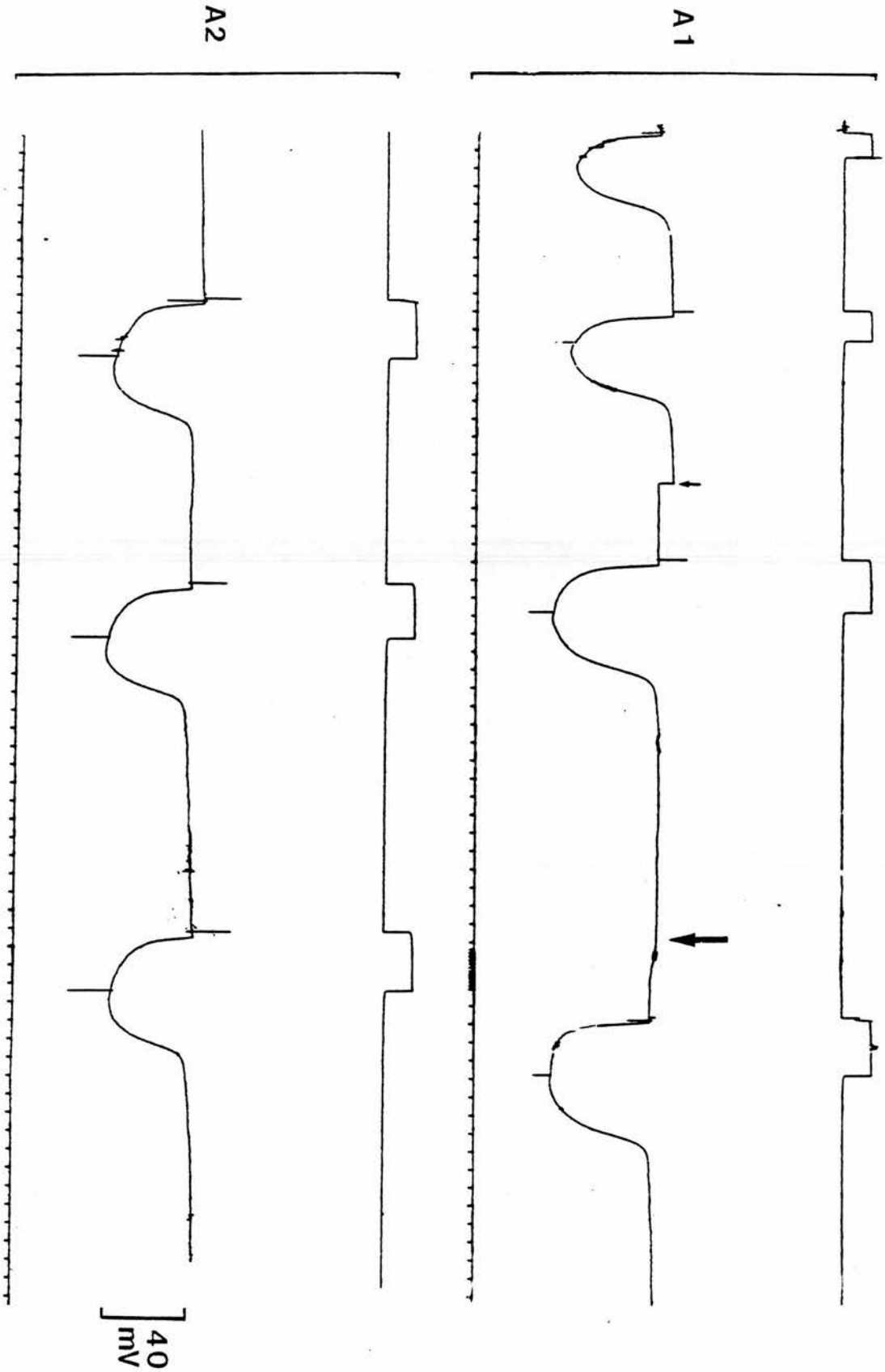
The presence of high concentrations of magnesium (10 mM) or cobalt (5 mM) in the calcium-free solutions appeared to reduce successive responses to dopamine more rapidly than in solutions with no added divalent ions or with a low magnesium concentration (1 mM). This is illustrated in Figure 9 which shows the rapid decrease in responses to dopamine after more than an hour in a solution in which calcium was replaced by cobalt (5 mM).

## DISCUSSION

The results show a striking difference in the calcium requirement of transmitter release and that of the post-synaptic response to the putative neurotransmitter and strongly support the idea that dopamine acts by initiating release of calcium from a store which is of limited content but which may be readily replenished from the external medium.

FIGURE 8: Effect of reduction of calcium concentration in the bathing solution.

The control solution contained 5 mM calcium; the test solution contained 50µM calcium. Segments of a continuous recording are shown in A1 and A2. Bottom traces show 10 s time marks; in A1 chart speed was reduced for 2 min (thickened trace). Small arrow indicates 10 mV calibration step. A1: responses (middle trace) to dopamine ejected by prolonged 200 nA ionophoretic pulses (upper trace) in control solution which was changed at large arrow. The cell hyperpolarized by about 4 mV in test solution. A2: the maximum hyperpolarization during responses was unchanged.


A1
A2
40
mV

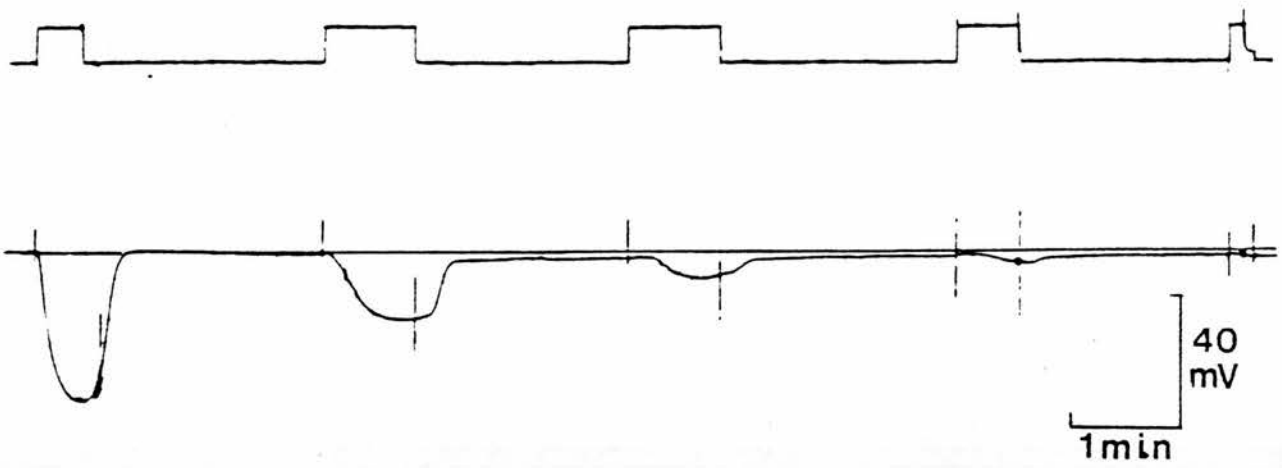



FIGURE 9: Effect of repeated dopamine stimulation in bathing solution with 'zero' calcium and 5 mM cobalt.

Continuous recording of responses (lower trace) to dopamine ejected by 100 nA ionophoretic currents of durations shown in upper trace. The preparation was left unstimulated in the bathing solution for 65 min before the recording started.

Responses to nerve stimulation were rapidly abolished after a ten-fold reduction in the external calcium ion concentration whereas application of dopamine, after a prolonged incubation of the salivary glands in calcium-free medium, could still produce hyperpolarizations. The responses to dopamine were dependent on calcium as they were reduced in calcium-free medium and were restored to their original magnitude on return to normal solution. The decrease in consecutive responses to a large charge of dopamine was only seen to occur in solutions without added calcium and this suggested that dopamine was causing depletion of a store of calcium. The calcium mobilized from such a store would augment the intracellular concentration thence activating potassium channels to produce a hyperpolarization (c.f. Section I, Part 1). Depletion of the store did not occur at concentrations as low as 50 μM suggesting that it could be refilled from the external solution and was saturated at very low calcium concentrations. It has also been reported that noradrenaline causes contraction of rabbit aorta in calcium-free medium by mobilizing calcium from a store which may be replenished from the external solution (Karaki, Kubota and Urakawa, 1979).

Hyperpolarizing responses due to an increased potassium conductance have been observed in calcium-free media elsewhere, e.g. in Aplysia neurones in responses to dopamine (Ascher, personal communication), 5-hydroxytryptamine

(Gerschenfeld and Paupardin, 1973) and acetylcholine (Kehoe, 1973). In the smooth muscle of the guinea-pig taenia coli the hyperpolarization produced by the action of adrenaline on α receptors is gradually reduced in calcium-free solution and Bülbring and Tomita (1970) have proposed the existence of an intracellular storage site for calcium in this tissue. Nishiyama and Petersen (1974) also realised the possibility of the involvement of a calcium store which would mediate the action of acetylcholine in producing hyperpolarizing responses in submaxillary glands bathed in medium depleted of calcium. Putney (1977) has used $^{86}Rb^{+}$ as a marker for calcium-induced potassium release in the parotid gland and showed that although sustained agonist-evoked release required the presence of extracellular calcium, a transient release of $^{86}Rb^{+}$ was evoked in calcium-free medium. The store of calcium in the parotid, like that of the cockroach salivary glands, was found to be inaccessible to EGTA.

In any of the 'zero' calcium solutions the hyperpolarization evoked by a small pulse of dopamine was more attenuated than that evoked by a large pulse. A similar effect has been described in pancreatic acinar cells where calcium deprivation resulted in a decrease in submaximal but not maximal responses to acetylcholine (Iwatsuki and Petersen, 1977). These observations may be explained by assuming that an increase in the agonist concentration results in activation of more receptors which,

in turn, allows mobilization of more calcium stores in the acinus. In the rat parotid (Marier, Putney and Van de Walle, 1978) and lacrimal glands (Parod and Putney, 1978) different receptors appear to regulate the same calcium stores and this accounts for cross-receptor inactivation seen in calcium-free medium.

Direct evidence for the involvement of an increase in intracellular calcium ion concentration in the mediation of electrical responses to glandular cell stimulants has been provided by intracellular injection of calcium ions. In pancreatic acinar cells (Petersen and Iwatsuki, 1978) intracellular calcium depolarizes cells and produces responses similar to that evoked by acetylcholine or hormones while in lacrimal acinar cells (Iwatsuki and Petersen, 1978) intracellular injection of calcium evokes hyperpolarizations similar to those seen on application of acetylcholine or adrenaline to the external acinar membrane.

The more rapid reduction in the responses to consecutive applications of dopamine in solution in which calcium has been replaced by cobalt may be due to:

i) substitution by cobalt of calcium in the store;

ii) inhibition of release of calcium; or

iii) inhibition of calcium influx after mobilization from the store.

If cobalt displaces calcium from its store, then it can only do so after stimulation by dopamine, otherwise a very

much smaller response would be elicited on dopamine ionophoresis after a prolonged period in cobalt solution. The other two possibilities have been proposed to explain the inhibitory effect of cobalt on contraction of the rat rectum in calcium-free medium by high potassium or acetylcholine (Yoshida, 1977). In the salivary glands, inhibition of release alone would not account for the rapid decrease in the effect of dopamine as there would remain an appreciable amount of calcium in the store and the first few consecutive responses would be expected to be attenuated to the same degree. Likewise, inhibiton of calcium influx would not show the rapid run-down of responses, but further experiments would be required to investigate the action of cobalt more thoroughly.

The effects of calcium removal on the electrical events in glandular cells are related to the effects on secretion. Fluid secretion from the cockroach salivary glands is still evoked by dopamine after one hour in calcium-free medium (Smith and House, 1979). Secretion, although decreased, is seen in other glands in calcium-free medium, e.g. the pancreas (Petersen, 1978), the parotid (Petersen and Pedersen, 1974) and the submaxillary gland (Douglas and Poisner, 1963). In the salivary glands of the blowfly Calliphora, a transient increase in secretion is evoked by 5-hydroxytryptamine in calcium-free medium containing EGTA and this is also due to release of calcium from a store in the gland (Prince and Berridge, 1973).

Calcium efflux has been noted from pancreatic acinar cells in response to acetylcholine and pancreozymin (Matthews, Petersen and Williams, 1973), from blowfly salivary glands in response to cAMP and 5-HT (Prince, Berridge and Rasmussen, 1972) and from pancreatic islet cells by glucose or readmission of a high calcium concentration (Herchuelz, Couturier and Malaisse, 1980). These effects are also connected with mobilization of calcium from intracellular stores.

The site of the proposed store in the cockroach salivary glands is unknown but, taking into consideration the suggested association between the calcium store and the dopamine-activated receptor, it seems reasonable to suppose the store to be located in the cell or basement membranes. It is of interest to note that the purified acetylcholine receptor of the electric organs of electric fish has a high binding capacity for calcium ions, which are not easily displaced by EDTA (Chang and Neumann, 1976).

# SECTION I: PART 3

## Ionophore A23187: Calcium requirement and acinar cell hyperpolarization

Introduction

Further investigations of the role of calcium in the production of the hyperpolarization were carried out with the use of the ionophore A23187. A23187 is a carboxylic acid antibiotic originally obtained from cultures of Streptomyces chartreusensis (Chaney, Demarco, Jones and Occolowitz, 1974) and more recently synthesised by Evans, Sacks, Kleschick and Taber (1979). It has a high affinity for divalent ions (Pfeiffer, Reed and Lardy, 1974) but can transport monovalent ions such as sodium (Flatman and Lew, 1977) and potassium (Pfeiffer and Lardy, 1976) in the absence of divalent ions in the medium. Studies of the ionophore-calcium complex using absorption and fluorescent spectra (Pfeiffer et al, 1974) and conductance measurements (Case, Vanderkooi and Scarpa, 1974) show that the complex is neutral, having a stoichiometry of 2 : 1. The carboxylic groups of the two ionophore molecules may allow exchange of two protons for one calcium ion and Deber and Pfeiffer (1976) have suggested a conformation such that the cation is shielded by the ionophore molecules from the solvent, the exterior of the complex being hydrophobic, thus allowing the complex to pass into biological membranes.

In the present experiments the ionophore has been found to produce a prolonged hyperpolarization due to an increase in potassium permeability. A23187 is able to evoke a small hyperpolarization in the absence of calcium ions in the external solution but a substantial, sustained

effect is normally produced only in the presence of extracellular calcium. The results support the idea that an increase in potassium permeability results from an increase in the intracellular calcium ion concentration (c.f. Section I, Part 1) and that the ionophore can produce a response in the absence of extracellular calcium due to mobilization of a limited store of calcium in the acinus (c.f. Section I, Part 2).

## METHODS

These experiments were carried out using apparatus different from that previously described. The preparation was mounted so that the acini were stretched over a thin glass plate in a perspex chamber (volume, 2 ml) while the ducts and reservoirs were pinned to silicone resin surrounding the plate (see Figure 1). Electrodes (40 - 60 MΩ), made from glass capillaries (outer diameter, 1.2 mm) were filled with 3M KCl as before (see General Methods). These were inserted into acini using a Leitz micromanipulator, and vision was much improved by the use of a Zeiss Jena microscope (x 320 magnification) and Nomarski Optics. The glands were superfused (2 ml $min^{-1}$) with a solution of the same composition as previously used, viz. (mM): NaCl 160, $CaCl_2$ 5, KCl 1, Tns-HCl buffer pH 7.6, 5; and any modifications of the fluid contents are specified in the text. A23187 (Eli-Lilly) was dissolved (0.5 - 1 mg) in 1 ml of methanol and added to the bathing solution to give

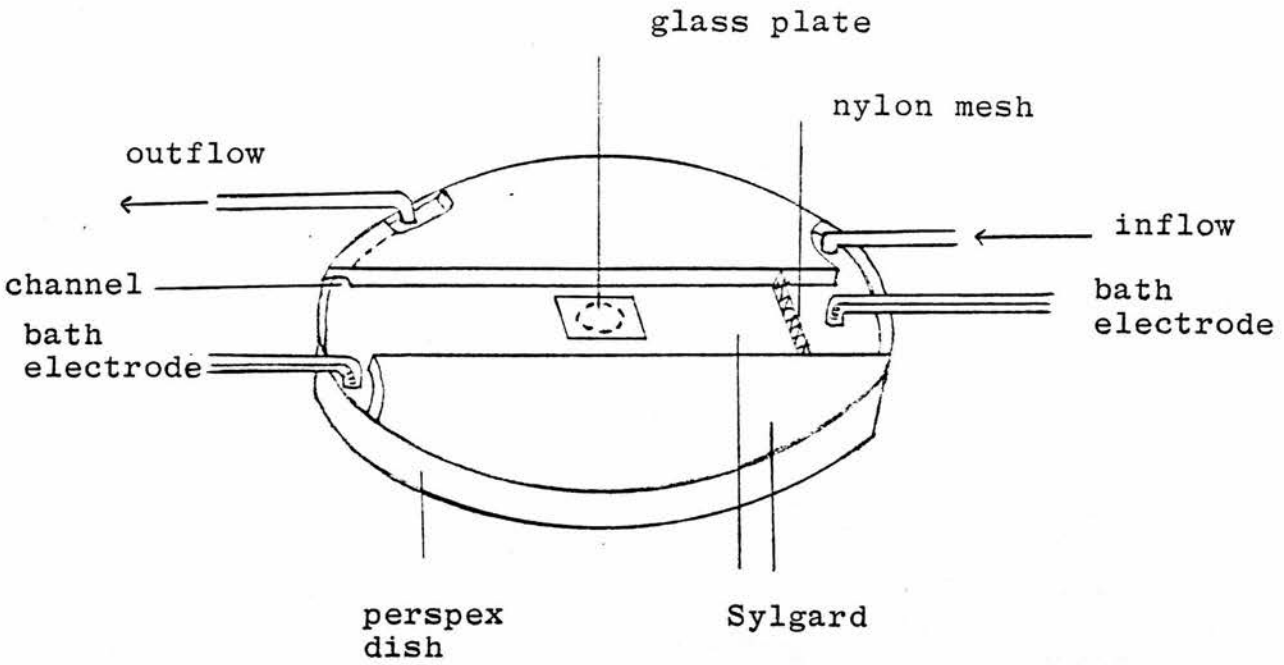



FIGURE 1: Diagram of experimental chamber.

a final concentration of 5 - 10 μg $ml^{-1}$. 1% methanol had no effect on the membrane potential. Membrane potentials were recorded as before.

RESULTS

Figure 2 shows that the addition of A23187 to the bathing solution (containing 5 mM calcium) produced a sustained hyperpolarization. Phentolamine ($10^{-4}$M), which has been shown to antagonise the hyperpolarization due to ionophoretically applied dopamine and nerve stimulation (Ginsborg et al, 1976; Bowser-Riley et al, 1978), did not reverse this effect suggesting that the ionophore is acting directly on the acinar cell and not by releasing transmitter from the nerve endings (Thoa, Costa, Moss and Kopin, 1974). The effect of A23187 on the resting potential of a number of cells is shown in Table 1.

It seems probable that the hyperpolarization observed after A23187 incubation is due to an increase in membrane potassium conductance, and, although this has not been demonstrated directly, the idea is supported by investigations of the effects of changes in extracellular potassium concentration on membrane potentials of cells in the hyperpolarized state. In the experiment shown in Figure 3, a prolonged hyperpolarization was induced by A23187 and an increase in the external potassium concentration from 1 mM to 5 mM resulted in a depolarisation of 22 mV. In six cells (mean resting potential -46 mV) in untreated

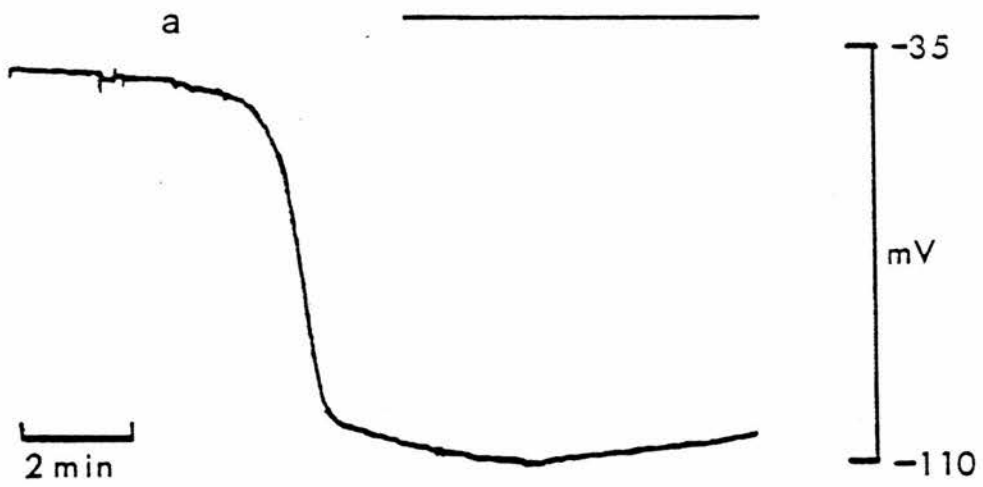



FIGURE 2: Effect of ionophore A23187 on the membrane potential of the cockroach salivary gland acinar cell.

A23187 (5 μg $ml^{-1}$) was applied in bathing solution containing 5 mM calcium at a. On establishment of the hyperpolarization, phentolamine ($10^{-4}$M) was also added to the perfusing solution and was present during the period shown by the bar.

TABLE 1: The effect of ionophore A23187 on the resting potential of salivary gland acinar cells in the presence of 5 mM calcium.

| Resting potential (mV) | Hyperpolarization (mV) in the presence of A23187 (5 μg $ml^{-1}$) |
|---|---|
| -45 | 50 |
| -69 | 30 |
| -66 | 24 |
| -74 | 13 |
| -66 | 30 |
| -40 | 34 |
| -74 | 15 |
| -64 | 20 |
| -56 | 25 |
| -38 | 72 |
| -42 | 68 |
| -45 | 65 |
| -49 | 22 |
| -47 | 4 |
| -62 | 45 |
| -38 | 72 |
| -50 | 60 |
| Mean ± SE -54 ± 3 | 38 ± 5 |

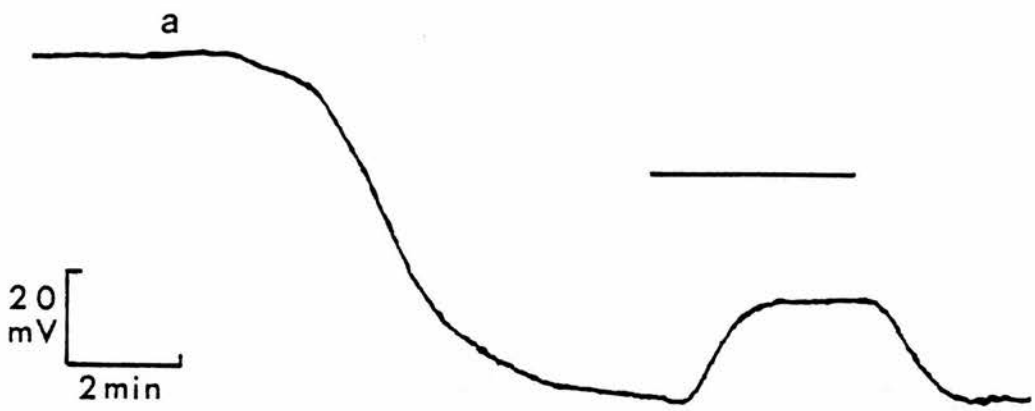



FIGURE 3: The effect of changing the external potassium ion concentration on the hyperpolarization evoked by the ionophore.

A23187 (5 μg $ml^{-1}$) was present throughout the experiment from a. The external potassium ion concentration was increased from 1 mM to 5 mM over the period shown by the bar.

preparations, a five-fold increase in potassium concentration produced varying changes in membrane potential, viz. (mV) +10, +10, +9, 0, 0, -10 (mean + 3 mV) whereas in each of four ionophore-treated preparations augmentation of the potassium concentration resulted in a marked depolarisation (mean = 20 mV). This comparison is consistent with the idea that the ionophore produces an increase in membrane potassium permeability.

Calcium-free medium

In Figure 4, removal of calcium from the bathing solution during incubation with A23187 (5 μg $ml^{-1}$) resulted in a complete reversal of the ionophore-induced hyperpolarization. This suggests that the prolonged increase in membrane potential is dependent on the presence of extracellular calcium ions. Nevertheless, the ionophore is capable of producing a hyperpolarization in the absence of extracellular calcium ions (Table 2), although, in general, the effects are smaller and shorter lasting than in the presence of calcium. Three examples are illustrated in Figure 5. Cells were impaled during incubation in calcium-free solution and introduction of the ionophore (5 μg $ml^{-1}$) produced hyperpolarizations having peak values of 3, 10 and 28 mV. Addition of 5 mM calcium in the presence of the ionophore produced larger and more prolonged hyperpolarizations with maximum amplitudes of 45, 32 and 52 mV, respectively. Although the large

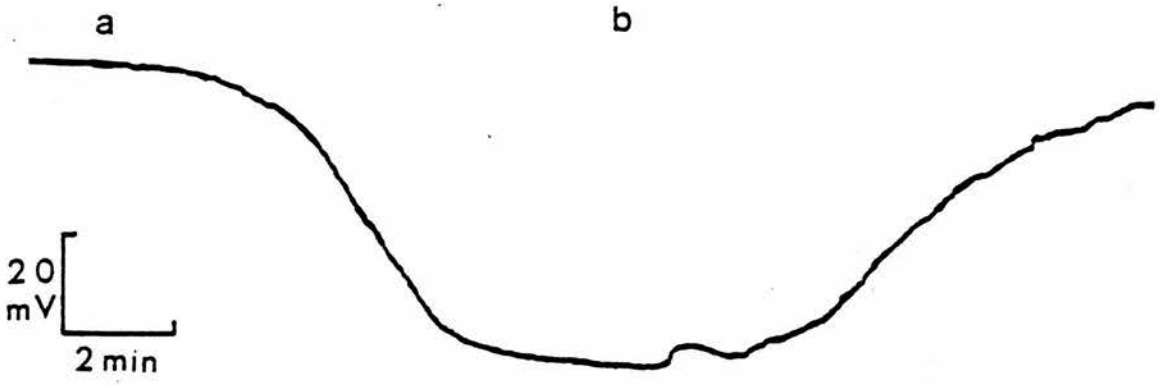



FIGURE 4: The effect of calcium removal on the response to A23187.

At a, ionophore (5 μg $ml^{-1}$) was added in normal solution, containing 5 mM calcium. From b, the ionophore was present in bathing solution without added divalent ions.

TABLE 2: The effect of ionophore A23187 on the resting potential of salivary gland acinar cells in the absence of added divalent ions (sustained effect shown by *).

| Resting potential (mV) (no added divalent ions) | Hyperpolarization (mV) in the presence of A23187 (5 μg $ml^{-1}$) |
|---|---|
| -30 | 0 |
| -25 | 3 |
| -29 | 33 |
| -48 | 3 |
| -40 | 10 |
| -30 | 0 |
| -32 | 6 |
| -42 | 30 |
| -34 | 28* |
| -43 | 5 |
| -34 | 0 |
| -36 | 38 |
| -26 | 3 |
| -50 | 50 |
| -36 | 0 |
| Mean ± SE -36 ± 2 | 14 ± 4 |

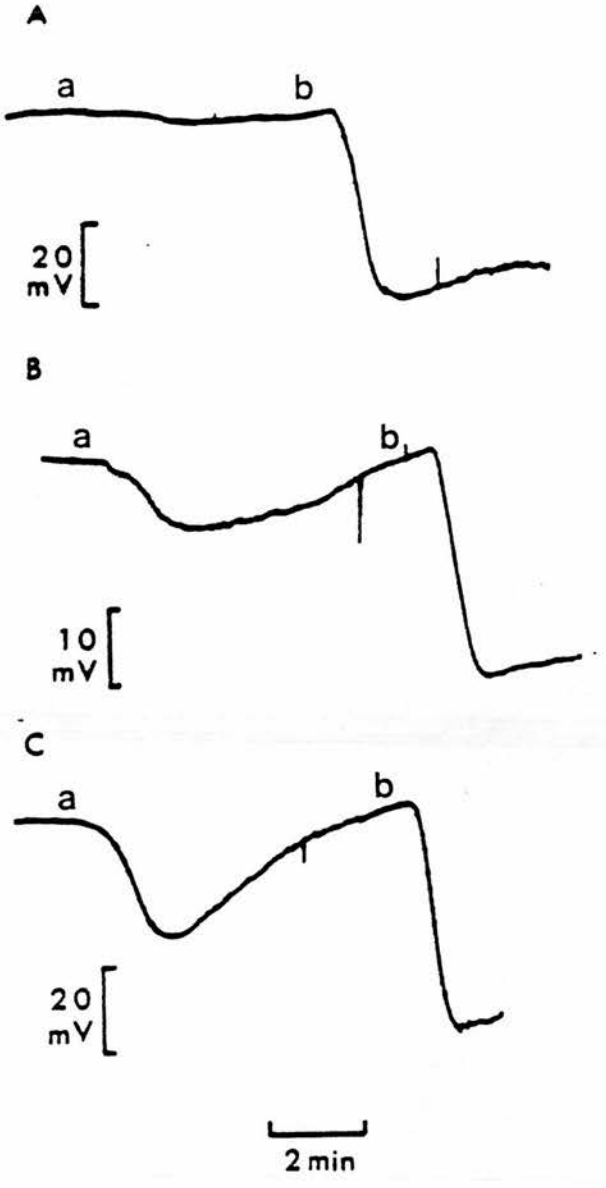



FIGURE 5: Three examples of the effects of ionophore in the absence of extracellular calcium ions.

The preparations were incubated in calcium-free medium for 20 min before introduction of A23187 (5 μg $ml^{-1}$) at a. 5 mM calcium was added to the solution at b.

responses on introduction of calcium may be partly due to a calcium readmission response (Section I, Part 1) in addition to any ionophoretic effect, they do show that the smaller values in calcium-free solution were not due to the inability of the cells to produce a large increase in the internal negativity. The resting potentials in the calcium-free media (Table 2) were generally less than those recorded in calcium containing solutions (Table 1).

Magnesium containing solution

A23187 also has a high affinity for magnesium ions (Pfeiffer et al, 1974). The introduction of 5 mM magnesium in the presence of ionophore (5 $\mu g\ ml^{-1}$) after incubation in calcium-free medium produced small (4 - 12 mV) hyperpolarizations (Figure 6) which were transient or prolonged. Similar results were obtained when magnesium was added in the absence of ionophore suggesting that this is a 'readmission' phenomenon, reminiscent of that seen on addition of calcium ions to calcium deficient solution (Section I, Part 2), rather than an ionophoretic effect.

Prolonged action of A23187

In the presence of extracellular calcium ions, cells remain hyperpolarized after removal of ionophore from the bathing solution. In Figure 7, the preparation was incubated in a solution which contained both A23187 (10 $\mu g\ ml^{-1}$) and 5 mM calcium and after 5 min the ionophore

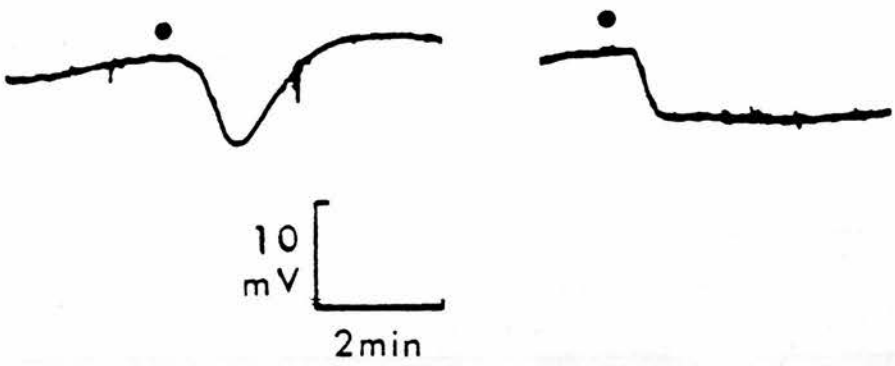



FIGURE 6: A23187 and magnesium.

The preparations were incubated in calcium-free medium for 15 min and at • ionophore (5 μg $ml^{-1}$) in the presence of 5 mM $MgCl_2$ was added.

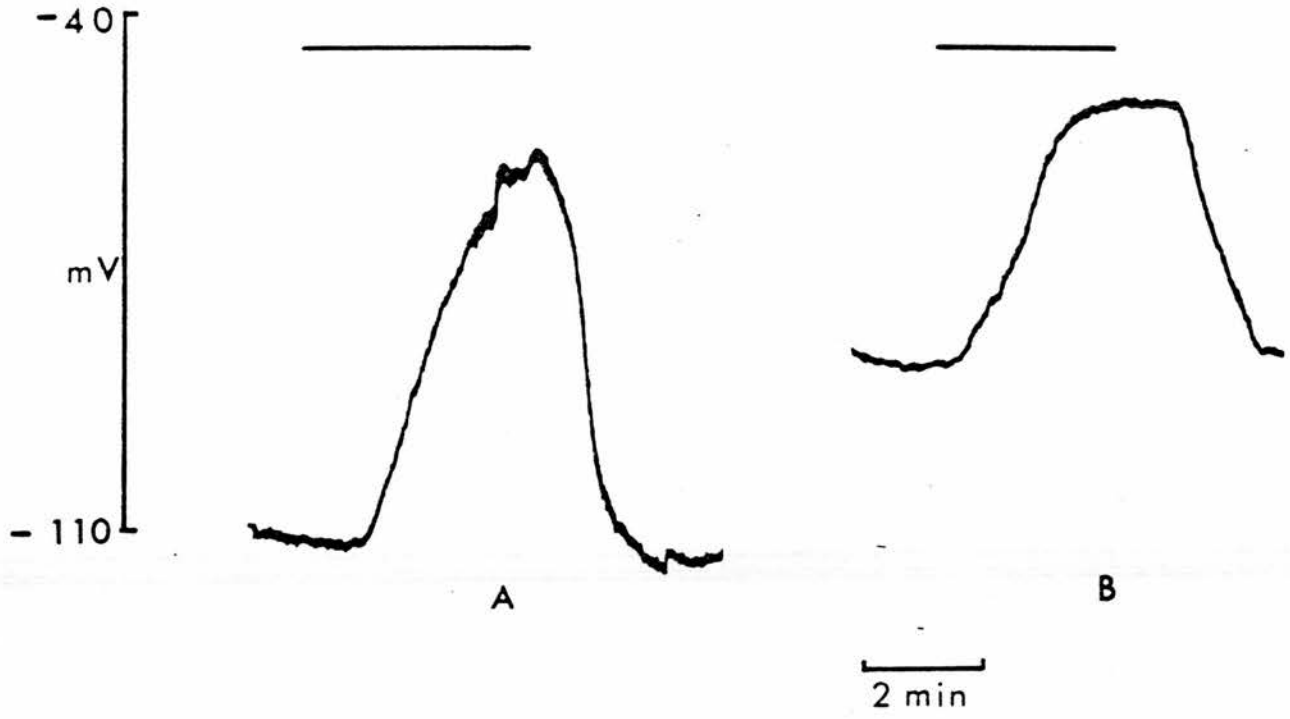



FIGURE 7: The prolonged action of ionophore.

The preparation was incubated in A23187 (10 μg $ml^{-1}$) in calcium containing solution for 5 min, then bathed in normal solution without ionophore. After 10 min in ionophore-free medium cell A was impaled and after 40 min cell B was impaled. 5 mM calcium was replaced by 1 mM magnesium + 1 mM EGTA for 4 min in A and for 3 min in B (shown by the bars).

was washed out. Ten minutes later a cell (A) was impaled and found to have a potential of -110 mV as compared to an average value of -68 mV in 3 cells before incubation in A23187. Replacement of calcium by 1 mM $Mg^{2+}$ + 1 mMEGTA resulted in a rapid depolarization (as in Figure 4) to a lower internal negativity and on returning to the calcium containing solution, without ionophore, the cell reverted to its hyperpolarized state. Another cell (B) was impaled 25 min later and found to have a resting potential of -83 mV, which was also reversibly reduced by calcium removal. This experiment indicates that the effect of the ionophore remains after washout of the solution containing the ionophore.

## DISCUSSION

Many of the biological changes produced by ionophore A23187 can be attributed to its calcium translocation properties and are dependent on the presence of extracellular calcium ions (see Table 3 for examples). However, some of the effects produced by the ionophore are not dependent on the presence of calcium in the bathing solution and appear to be due instead to mobilization of intracellular calcium (see Table 4).

The present results show that A23187 acts directly on the acinar cell membrane and not on release of neurotransmitter and that, although its action in the presence of calcium is more pronounced, it is able to produce a

TABLE 3: Calcium dependent effects of A23187.

| Effect | Reference |
|---|---|
| ↑$K^+$ efflux from salivary glands | Selinger, Eimerl & Schramm, 1974 |
| ↑enzyme secretion from salivary glands | Rossignol, Herman & Chambaut, 1974; Butcher, 1975 |
| ↑enzyme release from the exocrine pancreas | Eimerl, Savion, Heichal & Selinger, 1974; Williams & Lee, 1974; Schreurs, Swartz, DePont & Bonting, 1976; Poulsen & Williams, 1977 |
| ↑glucagon release from the endocrine pancreas | Ashby & Speake, 1975; Wollheim, Blondel, Renold & Sharp, 1976 |
| ↑histamine secretion from mast cells | Foreman, Mongar & Gomperts, 1973; Cochrane & Douglas, 1974 |
| ↑catecholamine secretion from the adrenal medulla | Cochrane, Douglas, Mouri & Nakazato, 1975; Garcia, Kirpekar & Prat, 1975 |
| mobilization of phospholipids in erythrocytes and polymorphonuclear leukocytes (PMNL) | Dise, Lake, Goodman & Rasmussen, 1976; Borgeat & Samuelsson, 1979 |
| releases prostaglandins from PMNL | Wentzell & Epand, 1978 |
| ↓cell agglutination by inducing microtubule destruction | Hart, Fisher & Hallinan, 1976; Poste & Nicholson, 1976 |
| ↑glucose transport in thymocytes | Reeves, 1975 |
| ↑fertilizing capacity of spermatozoa | Reyes, Goicoechea & Rosado, 1978 |
| ↓action potential duration and hyperpolarizes the membrane potential in Purkinje fibres | Gelles, 1977 |
| ↑cardiac contractility | Pressman, 1973; Holland, Steinberg & Armstrong, 1975 |
| contracts intestinal smooth muscle | Swamy, Ticku, Triggle & Triggle, 1975 |
| releases dopamine from synaptosomes | Holtz, 1975 |
| releases 5-HT from brain slices | Elks, Youngblood & Kizer, 1979 |

(↑ = increases, ↓ = decreases)

TABLE 4: Effects produced by the ionophore in the absence of external calcium.

| Effect | Reference |
|---|---|
| ↑insulin secretion from the pancreas | Ashby & Speake, 1975; Charles, Lawecki, Pictet and Grodsky, 1975; Karl, Zawalich, Ferrendelli & Matschinsky, 1975 |
| ↓ATP in erythrocytes and ↑ potassium efflux | Kirkpatrick, Hillman & LaCelle, 1975; Reed, 1976 |
| ↑secretion from platelets | Feinman & Detwiler, 1974; Feinstein & Fraser, 1975; Murer, Stewart, Rausch & Day, 1975; Warner & Brossmer, 1975 |
| ↑potassium and calcium efflux from hepatocytes | Burgess, Claret & Jenkinson, 1979 |
| ↑prostaglandin and thromboxane synthesis in platelets | Knapp, Oelz, Roberts, Sweetman, Oates & Reed, 1977 |
| activates unfertilized eggs | Steinhardt, Epel, Carroll & Yanagimachi, 1974; Schroeder & Strickland, 1974 |
| ↑calcium extrusion from intracellular organelles | Reed & Lardy, 1972; Scarpa, Baldassare & Inesi, 1972; Desmedt & Hainaut, 1979 |

hyperpolarization in the absence of calcium. The prolonged increase in membrane potential must therefore be due to an influx of calcium ions via the ionophore. This is similar to the prolonged hyperpolarization induced after incubation of the salivary glands in calcium-free, cobalt medium (Part 1) in that an influx of calcium from the external solution appears to be required. The transient effect sometimes seen in calcium-free media may occur as a result of the ionophore releasing calcium from a store in the acinus; this is consistent with the proposal in the previous section that there is a calcium store which can be mobilized by dopamine to produce responses in acinar cells bathed in zero calcium solution. Berridge (1975) has also reported a transient increase in fluid secretion by isolated salivary glands of the blowfly on exposure to A23187 in calcium-free solution due to mobilization of intracellular calcium. Sustained secretion from the blowfly salivary gland required the presence of calcium as does the prolonged increase in membrane potential in the cockroach salivary gland.

The hyperpolarization due to the ionophore is dependent on the external potassium ion concentration and is consistent with the hypothesis that an increase in the intracellular calcium ion concentration activates potassium channels in the cockroach salivary glands (c.f. Part 1). Calcium-dependent activation of potassium channels has been reported in a variety of cells (see Meech, 1978) and

Atwater (1980) has suggested that A23187-induced influx of calcium activates an increase in potassium permeability in mouse pancreatic β-cells.

The ionophore has also been found to have a prolonged action as has been shown for catecholamine release from the adrenal medulla (Garcia, Kirpekar and Prat, 1975) and muscle contraction (Devore and Nastuk, 1977). The lipophilic nature of the molecular conformation in the presence of calcium, as proposed by Deber and Pfeiffer (1974), presumbably results in the ionophore remaining in the cell membranes after its removal from the bathing solution.

The resting potentials of acinar cells obtained in these experiments (mean -54 mV, Table 1) were somewhat greater than those reported previously in solutions containing 5 mM calcium and 1 mM potassium (-40 mV; House, 1973). Similarly, in parotid (Pedersen and Petersen, 1973) and submaxillary (Kagayama and Nishiyama, 1974) glands, larger resting potentials were recorded in later experiments. Here, the difference may be associated with the use of higher resistance microelectrodes or with better visualization of the cells and, thus, the ability to sample a larger population. The large range in resting potentials (-30 to -80 mV), which can now be recorded, may reflect differences in the permeabilities of the cell membranes, possibly associated with variations in their secretory activity.

In calcium-free media the resting potentials were much lower (mean = -36 mV) than those recorded in calcium-containing media (mean = -54 mV). In order to determine what changes in permeability occur during incubation in the calcium-free media, it would be necessary to measure changes in input resistance (c.f. discussion in Section I, Part 1).

The action of magnesium to increase the membrane potential after incubation in calcium-free solution is independent of the presence of the ionophore. The hyperpolarization is analogous to the 'readmission' response seen with calcium but tends to be much smaller and may be due to displacement of calcium from the acinar cell membrane as has been suggested by Petersen and Ueda (1976) in pancreatic acinar cells.

# SECTION II

## RECEPTOR LOCALIZATION, BENZTROPINE AND ADENYLATE CYCLASE

## Introduction

This section is a report of three short investigations concerned with:

1. receptor localization;
2. presynaptic uptake inhibition; and
3. adenylate cyclase.

The experiments in each of the sections are not extensive but are sufficiently interesting to be reported here. Further work may add to the information received from these investigations and may allow more definite conclusions to be made.

# SECTION II: PART 1

## Intracellular and extracellular ionophoresis of agonist in the salivary glands.

## Introduction

It is generally accepted that receptors to neurotransmitters are located on the outer surface of cell membranes and there is good evidence to support this idea, e.g. in striated muscles (Del Castillo and Katz, 1955), in cardiac Purkinje fibres (Reuter, 1974), and in pancreatic acinar cells (Iwatsuki and Petersen, 1977). In the experiments reported in this section, intracellular application of agonists has been found to produce responses similar to that seen on stimulation of the nerve or on extracellular ionophoresis of agonist.

## METHODS (see General Methods)

In these experiments the recording electrode contained dopamine HCl (0.05 - 0.7M) dissolved in 1M or 3M potassium acetate and was connected to an electrometer amplifier (W P Instruments) so that current could also be passed through the electrode to allow application of dopamine inside the same cell from which the recordings were made.

## RESULTS

Figure 1A shows two successive hyperpolarizations of an acinar cell elicited by nerve stimulation (N) and by a current pulse to the intracellular electrode (D) equivalent to a charge of 40 nC. Figure 1B shows the response to a burst of nerve stimulation (N) followed by three responses to dopamine ejected by current pulses of

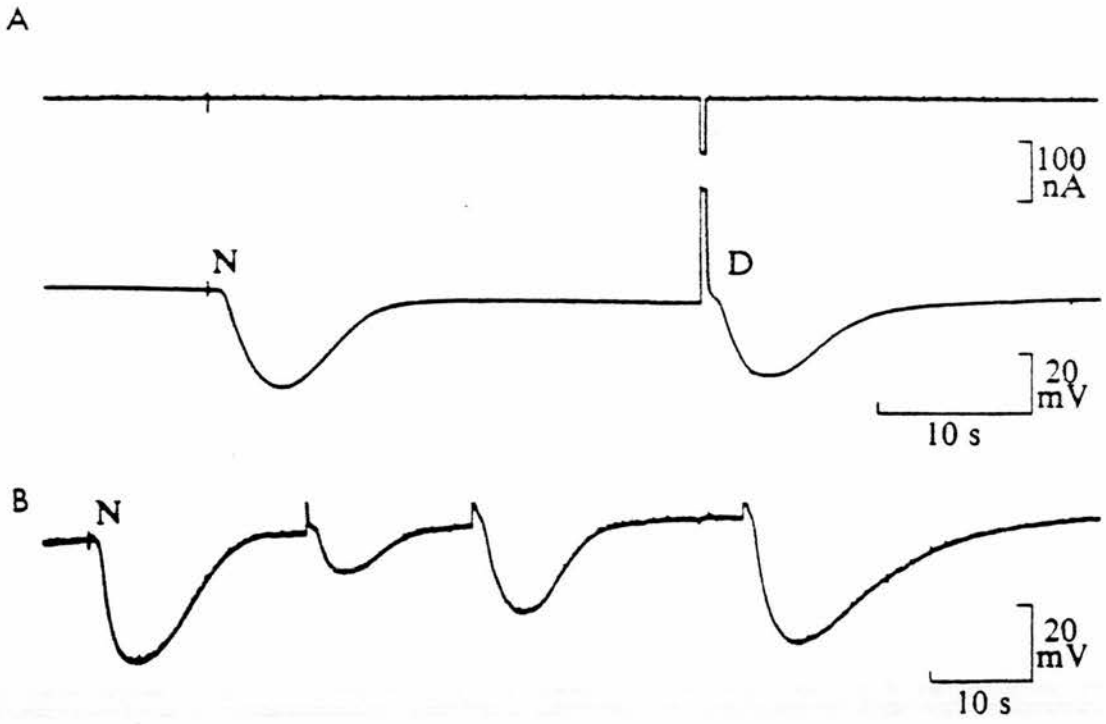



FIGURE 1: Hyperpolarizing responses to nerve stimulation and to intracellular application of dopamine.

1A, upper trace, current passed through the recording electrode; lower trace, voltage.
A single stimulus was applied to the salivary nerves at N and at D dopamine was ejected from the intracellular electrode by a 100 nA current for 0.4 s. The electrode contained 0.7M dopamine and 1M potassium acetate.

1B, responses to a burst of 3 nerve stimuli (N) and to 3 separate pulses of dopamine ejected from an intracellular electrode (containing 0.3M dopamine and 1M potassium acetate) by currents of 100 nA for durations of 0.2, 0.4 and 0.6 s respectively.

increasing magnitude, equivalent to 20, 40 and 60 nC respectively. The amplitude of the hyperpolarization is seen to increase with the ionophoretic charge.

Dopamine was applied intracellularly in about 100 cells and responses were obtained in approximately two-thirds of these. In a few experiments it was also found that adrenaline, noradrenaline and 5-hydroxytryptamine, all of which produce hyperpolarizations when applied extracellularly (Bowser-Riley and House, 1976), could produce responses on intracellular application.

The responses to intracellular and extracellular ionophoresis of dopamine have a similar latency and time course (Figure 2A, upper trace). In order to compare the sensitivity of a number of cells to the two methods of ionophoresis, the same electrode was used for extracellular and intracellular application in the following manner. A cell was first impaled with a recording electrode and when the ionophoretic pipette, containing 50 mM dopamine in 3M potassium acetate, was positioned close to the acinus a hyperpolarization was produced by a pulse of dopamine. The dopamine-containing pipette was then inserted into the acinus and was also used for recording if the first intracellular electrode was dislodged. Values for the ratio of response amplitude to ionophoretic charge were calculated. The maximum sensitivities for the two methods of ionophoresis were similar, viz. 3 mV $nC^{-1}$ for intracellular and 2.3 mV $nC^{-1}$ for extracellular dopamine application.

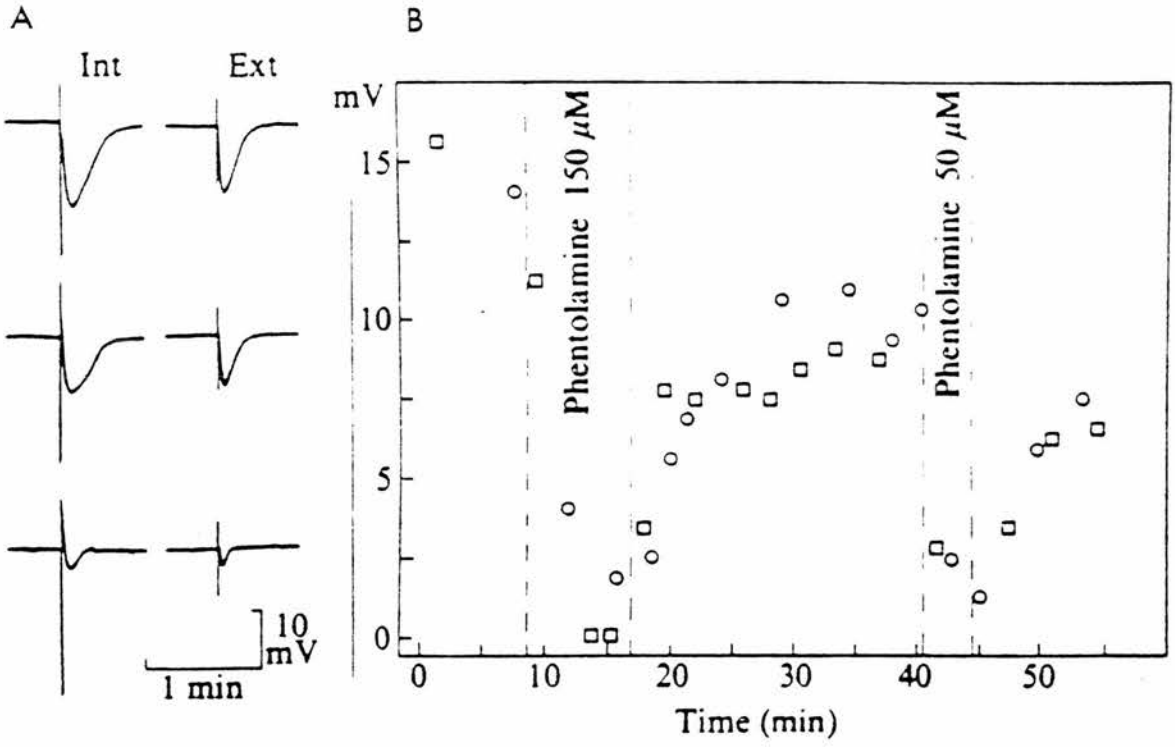



FIGURE 2: Effect of phentolamine on intracellularly recorded responses, elicited by intracellular and extracellular dopamine application.

Both the intracellular and extracellular micropipettes contained 0.5M dopamine and 3M potassium acetate.

2A, Int represents responses to impulses of dopamine ejected from the intracellular electrode (100 nA for 0.4s) and Ext represents responses to pulses of dopamine ejected from the extracellular pipette (200 nA for 0.08s).
Top records - control; middle records - during recovery from a short exposure to 150μM phentolamine; bottom records - in 50μM phentolamine.

2B, the time course of the experiment from which the records in 2A were taken.
o - responses to intracellularly applied dopamine;
□ - responses to extracellularly applied dopamine.

The effect of phentolamine, which is an antagonist of extracellularly applied dopamine (Ginsborg et al, 1976; Bowser-Riley et al, 1978), was compared on responses to extracellular and intracellular dopamine (Figure 2). An intracellular electrode, containing 0.5M dopamine in 3M potassium acetate, was used for intracellular dopamine ionophoresis and for recording responses evoked by the intracellular dopamine and by dopamine applied from a pipette close to the acinus. In Figure 2A the traces (from the top) show responses to intracellular and extracellular dopamine in (1) control solution, (2) control solution on washout of 150 μM phentolamine, and (3) in solution containing 50 μM phentolamine. Figure 2B shows the time course of the experiment of Figure 2A and it is evident that phentolamine reduced the responses to intracellular and extracellular dopamine to the same extent.

## DISCUSSION

The responses evoked by intracellular dopamine application could be due to activation of receptors present on the inside of the cell membrane or to activation of receptors on the extracellular cell membrane by dopamine which has leaked out of the cell. The sensitivities of an acinus to the two methods of application were not very different. If receptors are only present on the outer cell membrane then it might be supposed that the sensitivity to the intracellularly

applied dopamine would be smaller as most of the dopamine would be sequestered inside the cell and diffusion to the outside would be limited. It is not known whether the same amount of dopamine is ejected from the electrode at its two positions (intracellular and extracellular) for a given charge. Noradrenaline release from pipettes in saline has been compared to the release from the pipettes in brain tissue and although some authors (Candy, Boakes, Key and Worton, 1974) found no difference in release, others have reported that release in tissue is less than half that in saline (Hoffer, Neff and Siggins, 1971) for the same charge. Even assuming that the amount of dopamine released is the same in the two situations, it seems probable that release from the extracellular position close to the acinus would result in occupation of more receptors due to diffusion over the surface of the acinus than if limited diffusion occurred from the inside of the acinus via a 'leak' in the membrane. However, Blackman, Ginsborg and House (1979) have shown that the rate of rise of the ionophoretic dopamine response is not determined by the amount of dopamine ejected and suggested that there is a limiting rate of rise of response evoked by a particular concentration. If this concentration is exceeded the only effect which might be seen is a small reduction in the latency of the response. Also, if the maximum response is elicited by occupation of only a few receptors (Stephenson, 1956) then diffusion from the

interior of the acinus may allow enough dopamine to reach the receptors surrounding the area of leakage to produce a response not dissimilar in time course and magnitude to that evoked by extracellular dopamine application.

The experiments carried out with phentolamine suggested, but did not prove, that the leakage hypothesis might be correct. Phentolamine has been shown to reduce responses to nerve stimulation and bath-applied agonists (Bowser-Riley et al, 1978) presumably by an action on the extracellular membrane receptors, and its similar inhibitory capacity on responses to both intra- and extracellular dopamine tends to support the theory that leakage of dopamine does occur. However, if there are intracellular receptors present, then there is the possibility of leakage of phentolamine into the cells to produce inhibition.

Ultrastructural studies have shown that receptors span cellular membranes, e.g. the acetylcholine receptor from the Torpedo electric organ has antigenic sites (to which labelled antibodies may bind) on both sides of the membrane, the outer surface having a greater number than the internal surface (Tarrab-Hazdai, Geiger, Fuchs and Amsterdam, 1978; Strader, Revel and Raftery, 1979). It has also been reported that insulin may bind to receptors and be transported intracellularly (Schlessinger, Shechter, Willingham and Pastan, 1978; Bergeron, Sikstrom, Hand and Posner, 1979).

Although these studies may support the idea that there are intracellular receptor sites, they do not show whether these sites are functionally important, and, in the case of the insulin, the transport to the interior of the cell occurs some time after binding to the extracellular site and may not be related to the cellular actions of the hormone.

Philpott and Petersen (1979) have made an electrophysiological study of the effects of intracellular and extracellular peptide hormone application on pancreatic acinar cells and reported that depolarization generally occurred only on extracellular ionophoresis. However, in a few cells, intracellular application of hormone did result in depolarization and, as the resting potentials in these cells were found to be very low, it was suggested that leakage had occurred due to improper sealing of the membrane round the impaling microelectrode.

In the experiments reported here, there was no obvious relation between resting membrane potential and the ability of the cell to hyperpolarize on intracellular application of dopamine, and thus the question of the existence of intracellular receptor sites remains to be resolved.

# SECTION II: PART 2

## Benztropine

## Introduction

The antiparkinsonian drug, benztropine, has been reported to be a potent inhibitor of dopamine uptake into synaptosomal preparations from the rat corpus striatum (Coyle and Snyder, 1969). In this section benztropine has been used in the cockroach salivary glands; the results do not support the idea that benztropine inhibits uptake into the cockroach salivary nerves.

## METHODS AND RESULTS

Benztropine mesylate was dissolved in 'control' solution and glands were superfused at 6 ml $min^{-1}$. $10^{-5}$M benztropine was found to decrease matching responses to nerve stimulation and to ionophoretic dopamine to the same extent (Figure 1). At $10^{-4}$M, responses were abolished within 10 minutes and reappeared on washout of the benztropine.

## DISCUSSION

These results do not support the proposal that benztropine inhibits dopamine uptake, otherwise responses evoked by nerve stimulation (assuming that dopamine is the neurotransmitter) would be expected to increase in the presence of benztropine.

It has been pointed out, however, that the results obtained by Coyle and Snyder (1969), who measured $^{3}$H-dopamine uptake, may have been misinterpreted (Orlansky and Heikkila, 1974). The decrease in uptake of $^{3}$H-

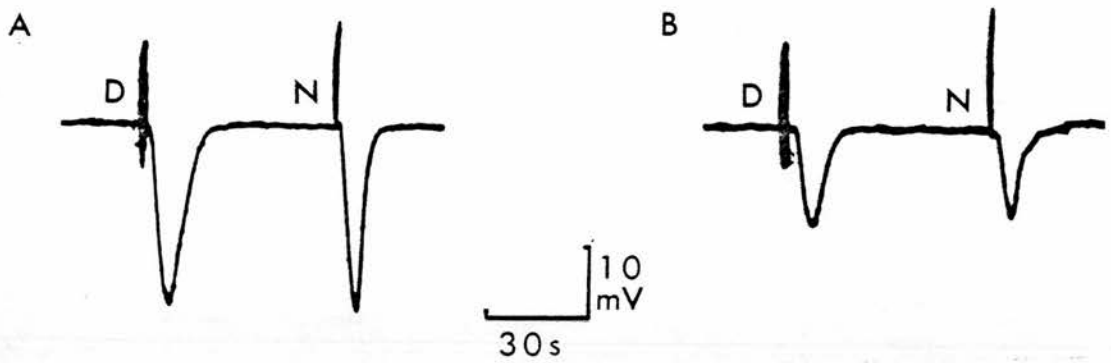



FIGURE 1: Effect of benztropine on responses to ionophoretic dopamine and to nerve stimulation.

At D dopamine was ejected from a microelectrode by a current of 100 nC and at N a stimulus was applied to the salivary nerves.

A: in 'control' solution.

B: in 'control' solution + $10^{-5}$M benztropine.

dopamine in the presence of benztropine would also be seen if benztropine actually stimulated release of dopamine, including $^{3}$H-dopamine taken up into striatal slices. Further experiments (Baumann and Maitre, 1976) showed that inhibition of uptake was directly related to dopamine depletion by benztropine and that the measurement of $^{3}$H-dopamine uptake into brain preparations was not a valid method for determining the inhibitory actions of drugs. The finding that benztropine does not affect uptake is consistent with the report by Broch (1979) that the uptake of 6-hydroxydopamine into nerve terminals, causing degeneration and dopamine depletion, is not blocked by benztropine but is blocked by desipramine, which appears to inhibit uptake.

If benztropine stimulated neurotransmitter release in the cockroach salivary glands, a hyperpolarization of the acinar cell membrane or potentiation of both nerve and dopamine responses would have been expected. It is not likely that the concomitant decrease in both responses was due to receptor desensitization since the prolonged presence of bath-applied dopamine does not decrease the magnitude of the hyperpolarization with time (Ginsborg et al, 1976). Therefore the possibility remains that benztropine inhibits responses postsynaptically and this may be related to its reported local anaesthetic activity (Wu and Narahashi, 1976).

# SECTION II: PART 3

## Adenylate Cyclase

## Introduction

Early studies of the action of dopamine in the brain suggested that the catecholamine acted by stimulating adenylate cyclase and thus increased the adenosine - $3^1$ : $5^1$ - monophosphate (cyclic AMP) concentration (Kebabian, Petzold and Greengard, 1972). It was therefore of interest to investigate whether dopamine action in the cockroach salivary glands was linked to stimulation of adenylate cyclase.

## METHODS AND RESULTS

Dibutyryl cAMP was dissolved in solution and glands superfused at 6 ml $min^{-1}$. No changes in membrane potential or in the responses to ionophoretic dopamine were observed in concentrations of dibutyryl cAMP as high as 1 mM.

A second experiment was carried out using 3-isobutyl-1-methylxanthine (IBMX, Aldrich Chemical Co.), which is 15 - 20 times more potent an inhibitor of the cAMP-hydrolytic enzyme, phosphodiesterase, then theophylline (Beavo, Rogers, Crofford, Hardman, Sutherland and Newman, 1970; Wells, Wu, Baird and Hardman, 1975). $10^{-6}$M IBMX had no effect either on the membrane potential or on responses to ionophoretic dopamine, while $10^{-4}$M IBMX sometimes inhibited responses to dopamine.

## DISCUSSION

These observations argue against the involvement of a dopamine-stimulated adenylate cyclase in the electrical responses of the salivary glands. Secretory experiments employing cAMP and theophylline have also indicated that dopamine does not act by stimulation of adenylate cyclase (C.R. House and R.K. Smith, personal communication).

Dopamine stimulation of adenylate cyclase has been reported in a number of tissues, e.g. brain (Kebabian et al, 1972), parathyroid gland (Brown, Carroll and Aurbach, 1977), retina (Brown and Makman, 1972) and molluscan ganglia (Cedar and Schwartz, 1972). The stimulation of the enzyme activity in these preparations has been estimated by direct measurement of the increase in cAMP in homogenates on exposure to dopamine, whereas the present experiments have investigated the effects of an increase in externally applied cAMP.

The indications here are that adenylate cyclase is not involved in the hyperpolarization of acinar cells; from the experiments of the previous section it appears that dopamine may act by increasing the intracellular calcium ion concentration. Therefore it may be suggested that the dopaminergic receptors in the salivary glands are different from those mentioned above. Indeed, dopamine does appear to act elsewhere without an increase in adenylate cyclase activity, e.g. anterior pituitary (Schmidt and Hill, 1977) but its mode of action has not been extensively investigated in such preparations.

# SUMMARY

I The role of calcium in electrical responses of the cockroach salivary glands.

1. Calcium ions are capable of producing a hyperpolarization of the acinar cells after a period of calcium deprivation.

2. This 'readmission' hyperpolarization is prolonged by prior incubation in calcium-free media containing cobalt.

3. The hyperpolarization is due to an increase in potassium permeability.

4. Dopamine produces a hyperpolarization of acinar cells in the absence of calcium. However, responses to repeated applications of the catecholamine decrease and are eventually abolished.

5. The ionophore, A23187, evokes a large, prolonged hyperpolarization associated with an increase in potassium permeability in calcium-containing media. It produces a smaller, transient response in calcium-free media.

6. The results suggest that an increase in intracellular calcium is sufficient to cause an increase in potassium permeability and that dopamine evokes hyperpolarization of acinar cells by mobilizing calcium from an acinar store. A23187 is also capable of producing a hyperpolarization by mobilizing calcium from the store.

II Short investigations

1. Dopamine elicits a hyperpolarization of cockroach salivary gland acinar cells on ionophoretic application intracellularly as well as extracellularly. The site of receptors is discussed.

2. Benztropine does not appear to affect uptake or release of neurotransmitter but does produce post-synaptic inhibitory effects.

3. Preliminary experiments suggest that stimulation of adenylate cyclase is not involved in the dopamine-evoked responses of Nauphoeta salivary glands.

# REFERENCES

AHLQUIST, R.P. (1948). A study of the adrenotropic receptors. Amer. J. Physiol. 153, 586-600.

ASHBY, J.P. and SPEAKE, R.N. (1975). Insulin and glucagon secretion from isolated Islets of Langerhans: the effects of calcium ionophores. Biochem. J. 150, 89-96.

ATWATER, I. (1980). Effects of the calcium ionophore, A23187, on glucose-induced electrical activity in mouse pancreatic β-cells. J. Physiol. (In Press).

BAKER, P.F., MEVES, H. and RIDGWAY, E.B. (1973). Effects of manganese and other agents on the calcium uptake that follows depolarization of squid axons. J. Physiol. 231, 511-526.

BARRETT, E.F. and BARRETT, J.N. (1976). Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium channel in frog motoneurones. J. Physiol. 255, 737-774.

BASSINGTHWAIGHTE, J.B., FRY, C.H. and McGUIGAN, J.A.S. (1976). Relationship between internal calcium and outward current in mammalian ventricular muscle; a mechanism for the control of action potential duration. J. Physiol. 262, 15-37.

BATZRI, S., SELINGER, Z. and SCHRAMM, M. (1971). Potassium ion release and enzyme secretion: adrenergic regulation by α- and β-receptors. Science 174, 1029-1031.

BATZRI, A., AMSTERDAM, A., SELINGER, Z., OHAD, I. and SCHRAMM, M. (1971). Epinephrine-induced vacuole formation in parotid gland cells and its independence of the secretory process. Proc. Nat. Acad. Sci. USA 68, 121-123.

BAUMANN, P.A. and MAITRE, L. (1976). Is drug inhibition of dopamine uptake a misinterpretation of *in vitro* experiments? Nature *264*, 789-790.

BEAVO, J.A., ROGERS, N.L., CROFFORD, O.B., HARDMAN, J.G., SUTHERLAND, E.W. and NEWMAN, E.V. (1970). Effects of Xanthine derivatives on lipolysis and on adenosine - 3': 5' - monophosphate phosphodiesterase activity. Mol. Pharm. *6*, 597-603.

BERGERON, J.J.M., SIKSTROM, R., HAND, A.R. and POSNER, B.I. (1979). Binding and uptake of $^{125}$I-insulin into rat liver hepatocytes and endothelium. J. Cell Biol. *80*, 427-443.

BERRIDGE, M.J. (1975). The interaction of cyclic nucleotides in the control of cellular activity. Adv. Cyclic Nuc. Res. *6*, pp. 30-34.

BERRIDGE, M.J. and FAIN, J.N. (1979). Inhibition of phosphatidylinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-hydroxytryptamine. Biochem. J. *178*, 59-69.

BERRIDGE, M.J., LINDLEY, B.D. and PRINCE, W.T. (1975). Membrane permeability changes during stimulation of isolated salivary glands of *Calliphora* by 5-hydroxytryptamine. J. Physiol. *244*, 549-567.

BERRIDGE, M.J. and PATEL, N.G. (1968). Insect salivary glands: stimulation of fluid secretion by 5-hydroxytryptamine and adenosine - 3',5' - monophosphate. Science *162*, 462-463.

BERRIDGE, M.J. and PRINCE, W.T. (1972). Transepithelial potential changes during stimulation of isolated salivary glands with 5-hydroxytryptamine and cyclic AMP. J. Exp. Biol. *56*, 139-153.

BLACKMAN, J.G., GINSBORG, B.L. and HOUSE, C.R. (1979). On the time course of the electrical response of salivary gland cells of Nauphoeta cinerea to ionophoretically applied dopamine. J. Physiol. 287, 81-92.

BLACKMAN, J.G., GINSBORG, B.L. and HOUSE, C.R. (1979a). On the effect of ionophoretically applied dopamine on salivary gland cells of Nauphoeta cinerea. J. Physiol. 287, 67-80.

BLAND, K.P. and HOUSE, C.R. (1971). Function of the salivary glands of the cockroach, Nauphoeta cinerea. J. Insect Physiol. 17, 2069-2084.

BLAND, K.P., HOUSE, C.R., GINSBORG, B.L. and LASZLO, I. (1973). Catecholamine transmitter for salivary secretion in the cockroach. Nature, New Biol. 244, 26-27.

BORGEAT, P. and SAMUELSSON, B. (1979). Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. Proc. Nat. Acad. Sci. USA 76, 2148-2152.

BOWSER-RILEY, F. (1978a). Release and actions of neurotransmitter molecules at neuroglandular junctions in cockroach salivary glands. Ph.D. Thesis, University of Edinburgh, pp. 1-18.

BOWSER-RILEY, F. (1978b). The salivary glands of the cockroach Nauphoeta cinerea (Olivier). Cell Tiss. Res. 187, 525-534.

BOWSER-RILEY, F. and HOUSE, C.R. (1976). The actions of some putative neurotransmitters on the cockroach salivary gland. J. Exp. Biol. 64, 665-676.

BOWSER-RILEY, F., HOUSE, C.R. and SMITH, R.K. (1978). Competitive antagonism by phentolamine of responses to biogenic amines and the transmitter at a neuroglandular junction. J. Physiol. 279, 473-489.

BRADING, A., BÜLBRING, E. and TOMITA, T. (1969). The effect of sodium and calcium on the action potential duration of the smooth muscle of the guinea-pig taenia coli. J. Physiol. 200, 637-654.

BREWARD, J., HOUSE, C.R. and SMITH, R.K. (1980). α-Flupenthixol: an antagonist of dopamine-evoked fluid secretion by an insect salivary gland preparation. Brit. J. Pharmacol. 69, 123-129.

BROCH, O.J. (1979). In vivo effects of benztropine and nomifensine on dopamine and noradrenaline terminals in rat brain. Eur. J. Pharmacol. 58, 419-424.

BROWN, E.M., CARROLL, R.J. and AURBACH, G.D. (1977). Dopaminergic stimulation of cyclic AMP accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. Proc. Nat. Acad. Sci. USA 74, 4210-4213.

BROWN, J.H. and MAKMAN, M.H. (1972). Stimulation by dopamine of adenylate cyclase in retinal homogenates and of adenosine - 3':5' - cyclic monophosphate formation in intact retina. Proc. Nat. Acad. Sci. USA 69, 539-543.

BÜLBRING, E. and TOMITA, T. (1969). Effect of calcium, barium and manganese on the action of adrenaline in the smooth muscle of the guinea-pig taenia coli. Proc. Roy. Soc. B. 172, 121-136.

BÜLBRING, E. and TOMITA, T. (1970). Effects of Ca removal on the smooth muscle of the guinea-pig taenia coli. J. Physiol. 210, 217-232.

BURGESS, G.M., CLARET, M., JENKINSON, D.H. (1979). Effects of catecholamines, ATP and ionophore A23187 on potassium and calcium movements in isolated hepatocytes. Nature 279, 544-546.

BUSIS, N.A. and WEIGHT, F.F. (1976). Spike after-hyperpolarization of a sympathetic neurone is calcium sensitive and is potentiated by theophylline. Nature 263, 434-436.

BUTCHER, F.R. (1975). The role of calcium and cyclic nucleotides in α-amylase release from slices of rat parotid: studies with the divalent cation ionophore A23187. Metabolism 24, 409-418.

CANDY, J.M., BOAKES, R.J., KEY, B.J. and WORTON, E. (1974). Correlation of the release of amines and antagonists with their effects. Neuropharmacology 13, 423-430.

CASE, G.D., VANDERKOOI, J.M. and SCARPA, A. (1974). Physical properties of biological membranes determined by the fluorescence of the calcium-ionophore A23187. Arch. Biochem. Biophys. 162, 174-185.

CEDAR, H. and SCHWARTZ, J.H. (1972). Cyclic adenosine monophosphate in the nervous system of Aplysia californica. II Effect of serotonin and dopamine. J. Gen. Physiol. 60, 570-587.

CHANEY, M.O., DEMARCO, P.V., JONES, N.D. and OCCOLOWITZ, J.L. (1974). The structure of A23187, a divalent cation ionophore. J. Amer. Chem. Soc. 96, 1932-1933.

CHANG, H.W. and NEUMANN, E. (1976). Dynamic properties of isolated acetylcholine receptor proteins: release of calcium ions caused by acetylcholine binding. Proc. Nat. Acad. Sci. USA, 73, 3364-3368.

CHARLES, M.A., LAWECKI, J., PICTET, R. and GRODSKY, G.M. (1975). Insulin secretion. Interrelationships of glucose, cyclic adenosine 3':5' - monophosphate and calcium. J. Biol. Chem. 250, 6134-6140.

COCHRANE, D.E. and DOUGLAS, W.W. (1974). Calcium-induced extrusion of secretory granules (exocytosis) in mast cells exposed to 48/80 or the ionophores A23187 and X537A. Proc. Nat. Acad. Sci. USA 71, 408-412.

COCHRANE, D.E., DOUGLAS, W.W., MOURI, T. and NAKAZATO, Y. (1975). Calcium and stimulus-secretion coupling in the adrenal medulla: contrasting stimulating effects of the ionophores X-537A and A23187 on catecholamine output. J. Physiol. 252, 363-378.

COYLE, J.T. and SNYDER, S.H. (1969). Antiparkinsonian drugs: inhibition of dopamine uptake in the corpus striatum as a possible mechanism of action. Science, 166, 899-901.

DEBER, C.M. and PFEIFFER, D.R. (1976). Ionophore A23187. Solution conformations of the calcium complex and free acid deduced from proton and carbon-13 nuclear magnetic resonance studies. Biochemistry 15, 132-141.

DEL CASTILLO, J. and KATZ, B. (1955). On the localization of acetylcholine receptors. J. Physiol. 128, 157-181.

DESMEDT, J.E. and HAINAUT, K. (1979). Dantrolene and A23187 ionophore: specific action on calcium channels revealed by the aequorin method. Biochem. Pharmacol. 28, 957-964.

DEVIS, G., SOMERS, G. and MALAISSE, W.J. (1977). Dynamics of calcium-induced insulin release. Diabetologia 13, 531-536.

DEVORE, D.I. and NASTUK, W.L. (1977). Ionophore-mediated calcium influx effects on the post-synaptic muscle fibre membrane. Nature, 270, 441-443.

DISE, C.A., LAKE, W.C., GOODMAN, D.B.P. and RASMUSSEN, H. (1976). Calcium-dependent stimulation of erythrocyte membrane phospholipid fatty acid incorporation by the ionophore A23187. J. Biol. Chem. 251, 4162-4164.

DOUGLAS, W.W. (1968). Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Brit. J. Pharmacol. 34, 451-474.

DOUGLAS, W.W. and POISNER, A.M. (1963). The influence of calcium on the secretory response of the submaxillary gland to acetylcholine or to noradrenaline. J. Physiol. 165, 528-541.

DOUGLAS, W.W. and RUBIN, R.P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol. 159, 40-57.

DREIFUSS. J.J., GRAU, J.D. and NORDMANN, J.J. (1973). Effects on the isolated neurohypophysis of agents which affect the membrane permeability to calcium. J. Physiol. 231, 96P-97P.

ECKERT, R. and TILLOTSON, D. (1978). Potassium activation associated with intraneuronal free calcium. Science 200, 437-439.

EIMERL, S., SAVION, N., HEICHAL, O. and SELINGER, Z. (1974). Induction of enzyme secretion in rat pancreatic slices using the ionophore A23187 and calcium. J. Biol. Chem. 249, 3991-3993.

ELKS, M.L., YOUNGBLOOD, W.W. and KIZER, J.S. (1979). Synthesis and release of serotonin by brain slices: effect of ionic manipulations and cationic ionophores. Brain Res. 172, 461-469.

EVANS, D.A., SACKS, C.E., KLESCHICK, W.A. and TABER, T.R. (1979). Polyether antibiotics synthesis. Total synthesis and absolute configuration of the ionophore A-23187. J. Amer. Chem. Soc. 101, 6789-6791.

FAIN, J.N. and BERRIDGE, M.J. (1979a). Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. Biochem. J. 178, 45-58.

FAIN, J.N. and BERRIDGE, M.J. (1979b). Relationship between phosphatidylinositol synthesis and recovery of 5-hydroxytryptamine-responsive $Ca^{2+}$ flux in blowfly salivary glands. Biochem. J. 180, 655-661.

FEINMAN, R.D. and DETWILER, T.C. (1974). Platelet secretion induced by divalent cation ionophores. Nature 249, 172-173.

FEINSTEIN, M.B. and FRASER, C. (1975). Human platelet secretion and aggregation induced by calcium ionophores. Inhibition by $PGE_1$ and dibutyryl cAMP. J. Gen. Physiol. 66, 561-581.

FLATMAN, P. and LEW, V.L. (1977). Does ionophore A23187 mediate Na transport in the absence of divalent ions? Nature 270, 444-445.

FOREMAN, J.C., MONGAR, J.L. and GOMPERTS, B.D. (1973). Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. Nature 245, 249-251.

FRANKENHAEUSER, B. and HODGKIN, A.L. (1957). The action of calcium on the electrical properties of squid axons. J. Physiol. 137, 218-244.

FRY, J.P., HOUSE, C.R. and SHARMAN, D.F. (1974). An analysis of the catecholamine content of the salivary gland of the cockroach. Brit. J. Pharmacol. 51, 116P-117P.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). Handb. Exp. Pharmak. 33, 283-335.

GAGE, P.W. and EISENBERG, R.S. (1969). Capacitance of the surface and transverse tubular membrane of frog sartorius muscle fibres. J. Gen. Physiol. 53, 265-278.

GARCIA, A.G., KIRPEKAR, S.M. and PRAT, J.C. (1975). A calcium ionophore stimulating the secretion of catecholamines from the cat adrenal. J. Physiol. 244, 253-262.

GÁRDOS, G. (1958). The function of calcium in the potassium permeability of human erythrocytes. Biochim. Biophys. Acta 30, 653-654.

GEDULDIG, D. and JUNGE, D. (1968). Sodium and calcium components of action potentials in the Aplysia giant neurone. J. Physiol. 199, 347-365.

GELLES, J.M. (1977). Use of calcium ionophores to determine the effects of intracellular calcium on the action potential of canine cardiac Purkinje fibres. Circulation Res. 41, 94-99.

GERSCHENFELD, H.M. and PAUPARDIN, D. (1973). Actions of 5-hydroxytryptamine on molluscan neuronal membranes. In Drug Receptors, ed. Rang, H.P. London: Macmillan, pp. 45-61.

GINSBORG, B.L. and HOUSE, C.R. (1976). The responses to nerve stimulation of the salivary gland of Nauphoeta cinerea Olivier. J. Physiol. 262, 477-487.

GINSBORG, B.L., HOUSE, C.R. and SILINSKY, E.M. (1974). Conductance changes associated with the secretory potential in the cockroach salivary gland. J. Physiol. 236, 723-731.

GINSBORG, B.L., HOUSE, C.R. and SILINSKY, E.M. (1976). On the receptors which mediate the hyperpolarization of salivary gland cells of Nauphoeta cinerea Olivier. J. Physiol. 262, 489-500.

GINSBORG, B.L., TURNBULL, K.W. and HOUSE, C.R. (1976a). On the actions of compounds related to dopamine at a neurosecretory synapse. Brit. J. Pharmacol. 57, 133-140.

GUERRERO, S. and RIKER, W.K. (1973). Effects of some divalent cations on sympathetic ganglion function. J. Pharm. Exp. Ther. 186, 152-159.

HACKETT, J.T. (1976). Selective antagonism of frog cerebellar synaptic transmission by manganese and cobalt ions. Brain Res. 114, 47-52.

HAGIWARA, S. and TAKAHASHI, K. (1967). Surface density of calcium ions and calcium spikes in the barnacle muscle fibre membrane. J. Gen. Physiol. 50, 583-601.

HAMMERSCHLAG, R., CHIU, A.Y. and DRAVID, A.R. (1976). Inhibition of fast axonal transport of {$^3$H} protein by cobalt ions. Brain Res. 114, 353-358.

HART, C.A., FISHER, D., HALLINAN, T. and LUCY, J.A. (1976). Effects of calcium ions and the bivalent cation ionophore A23187 on the agglutination and fusion of chicken erythrocytes by Sendai virus. Biochem. J. 158, 141-145.

HENQUIN, J.-C. and LAMBERT, A.E. (1975). Cobalt inhibition of insulin secretion and calcium uptake by isolated rat islets. Amer. J. Phsyiol. 228, 1669-1677.

HERCHUELZ, A., COUTURIER, E. and MALAISSE, W.J. (1980). Regulation of calcium fluxes in pancreatic islets: glucose-induced calcium-calcium exchange. Amer. J. Physiol. 238, E96-E103.

HIRST, G.D.S., HOLMAN, M.E. and SPENCE, I. (1974). Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. J. Physiol. 236, 303-326.

HOFFER, B.J., NEFF, N.H. and SIGGINS, G.R. (1971). Microiontophoretic release of norepinephrine from micropipettes. Neuropharmacology 10, 175-180.

HOLLAND, D.R., STEINBERG, M.I. and ARMSTRONG, W.McD. (1975). A23187: A calcium ionophore that directly increases cardiac contractility. Proc. Soc. Exp. Biol. Med. 148, 1141-1145.

HOLTZ, R.W. (1975). The release of dopamine from synaptosomes from rat striatum by the ionophores X537A and A23187. Biochim. Biophys. Acta 375, 138-152.

HOUSE, C.R. (1973). An electrophysiological study of neuroglandular transmission in the isolated salivary glands of the cockroach. J. Exp. Biol. 58, 29-43.

HOUSE, C.R. (1975). Intracellular recording of secretory potentials in a 'mixed' salivary gland. Experientia, 31, 904-906.

HOUSE, C.R. and GINSBORG, B.L. (1976). Actions of a dopamine analogue and a neuroleptic at a neuroglandular synapse. Nature, 261, 332-333.

HOUSE, C.R. and SMITH, R.K. (1978). On the receptors involved in the nervous control of salivary secretion by Nauphoeta cinerea Olivier. J. Physiol. 279, 457-471.

HUBBARD, J.I., JONES, S.F. and LANDAU, E.M. (1968). On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. J. Physiol. 194, 355-380.

ISENBERG, G. (1977a). Cardiac Purkinje fibres: resting, action and pacemaker potential under the influence of $\{Ca^{2+}\}_i$ as modified by intracellular injection. Pflügers Arch. 371, 51-59.

ISENBERG, G. (1977b). Cardiac Purkinje fibres: $\{Ca^{2+}\}_i$ controls steady state potassium conductance. Pflügers Arch. 371, 71-76.

IWATSUKI, N. and PETERSEN, O.H. (1977). Pancreatic acinar cells: localization of acetylcholine receptors and the importance of chloride and calcium for acetylcholine-evoked depolarization. J. Physiol. 269, 723-733.

IWATSUKI, N. and PETERSEN, O.H. (1977a). Pancreatic acinar cells: the acetylcholine equilibrium potential and its ionic dependency. J. Physiol. 269, 735-751.

IWATSUKI, N. and PETERSEN, O.H. (1977b). Acetylcholine-like effects of intracellular calcium application in pancreatic acinar cells. Nature 268, 147-149.

IWATSUKI, N. and PETERSEN, O.H. (1978). Intracellular $Ca^{2+}$ injection causes membrane hyperpolarization and conductance increase in lacrimal acinar cells. Pflügers Arch. 377, 185-187.

IWATSUKI, N. and PETERSEN, O.H. (1978a). Membrane potential, resistance, and intercellular communication in the lacrimal gland: effects of acetylcholine and adrenaline. J. Physiol. 275, 507-520.

JONES, L.M., COCKROFT, S. and MICHELL, R.H. (1979). Stimulation of phosphatidylinositol turnover in various tissues by cholinergic and adrenergic agonists by histamine and by caerulein. Biochem. J. 182, 669-676.

KAGAYAMA, M. and NISHIYAMA, A. (1974). Membrane potential and input resistance in acinar cells from cat and rabbit submaxillary glands *in vivo*: effects of autonomic nerve stimulation. J. Physiol. 242, 157-172.

KANAGASUNTHERAM, P. and RANDLE, P.J. (1976). Calcium metabolism and amylase release in rat parotid acinar cells. Biochem. J. 160, 547-564.

KARAKI, H., KUBOTA, H. and URAKAWA, N. (1979). Mobilization of stored calcium for phasic contraction induced by norepinephrine in rabbit aorta. Eur. J. Pharmacol. 56, 237-245.

KARL, R.C., ZAWALICH, W.S., FERRENDELLI, J.A. and MATSCHINSKY, F.M. (1975). The role of $Ca^{2+}$ and cyclic adenosine-3':5' - monophosphate in insulin release induced in vitro by the divalent cation A23187. J. Biol. Chem. 250, 4575-4579.

KATZ, B. (1966). In, Nerve, Muscle and Synapse, pp. 68-69. McGraw-Hill Book Company.

KEBABIAN, J.W., PETZOLD, G.I. and GREENGARD, P. (1972). Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain and its similarity to the "dopamine receptor". Proc. Nat. Acad. Sci. USA 69, 2145-2149.

KEHOE, J.S. (1973). Acetylcholine receptors in Aplysia neurones. In, Drug Receptors, Ed. Rang, H.P., pp. 63-85. London: Macmillan.

KESSEL, R.G. and BEAMS, H.W. (1963). Electron microscope observations on the salivary gland of the cockroach, Periplaneta americana. Z. Zellforschung 59, 857-877.

KIRKPATRICK, F.H., HILLMAN, D.G. and LA CELLE, P.L. (1975). A23187 and red cells: changes in deformability, $K^{+}$, $Mg^{2+}$, $Ca^{2+}$ and ATP. Experientia 31, 653-654.

KLEMM, N. (1972). Monoamine-containing nervous fibres in the foregut and salivary gland of the desert locust Schistocerca gregaria, Forskal (Orthoptera, Acrididae). Comp. Biochem. Physiol. 43A, 207-211.

KNAPP, H.R., OELZ, O., ROBERTS, L.J., SWEETMAN, B.J., OATES, J.A. and REED, P.W. (1977). Ionophores stimulate prostaglandin and thromboxane biosynthesis. Proc. Nat. Acad. Sci. USA 74, 4251-4255.

KOHLHARDT, M., BAUER, B., KRAUSE, H. and FLECKENSTEIN, A. (1973). Selective inhibition of the transmembrane Ca conductivity of mammalian myocardial fibres by Ni, Co and Mn ions. Pflügers Arch. 338, 115-123.

KRNJEVIĆ, K., LAMOUR, Y., MacDONALD, J.F. and NISTRI, A. (1978). Motoneuronal after-potentials and extracellular divalent cations. Can. J. Physiol. Pharmacol. 56, 516-520.

KRNJEVIĆ, K., LAMOUR, Y., MacDONALD, J.F. and NISTRI, A. (1979a). Effects of some divalent cations on motoneurones in cats. Can. J. Physiol. Pharmacol. 57, 944-956.

KRNJEVIĆ, K., LAMOUR, Y., MacDONALD, J.F., NISTRI, A., PUIL, E. and WERMAN, R. (1979b). Intracellular divalent cations and neuronal excitability. Can. J. Physiol. Pharmacol. 57, 957-972.

KRNJEVIĆ, K. and LISIEWICZ, A. (1972). Injections of calcium ions into spinal motoneurones. J. Physiol. 225, 363-390.

KRNJEVIĆ, K., PUIL, E. and WERMAN, R. (1975). Evidence for $Ca^{2+}$-activated $K^{+}$ conductance in cat spinal motoneurones from intracellular EGTA injections. Can. J. Physiol. Pharmacol. 53, 1214-1218.

KRNJEVIĆ, K., PUIL, E. and WERMAN, R. (1978). EGTA and motoneuronal after-potentials. J. Physiol. 275, 199-223.

MARIER, S.H., PUTNEY, J.W.Jr. and VAN DE WALLE, C.M. (1978). Control of calcium channels by membrane receptors in the rat parotid gland. J. Physiol. 279, 141-151.

MARTINEZ, J.R. and QUISSELL, D.O. (1976). Potassium release from the rat submaxillary gland in vitro. II Induction by parasympathomimetic secretagogues. J. Pharm. Exp. Ther. 199, 518-525.

MARTINEZ, J.R., QUISSELL, D.O. and GILES, M. (1976). Potassium release from the rat submaxillary gland in vitro. I Induction by catecholamines. J. Pharm. Exp. Ther. 198, 385-394.

MATTHEWS, E.K., PETERSEN, O.H. and WILLIAMS, J.A. (1973). Pancreatic acinar cells: acetylcholine-induced membrane depolarization, calcium efflux and amylase release. J. Physiol. 234, 689-701.

MAXWELL, D.J. (1978). Fine structure of axons associated with the salivary apparatus of the cockroach, Nauphoeta cinerea. Tissue & Cell 10, 699-706.

MEECH, R.W. (1974a). The sensitivity of Helix Aspersa neurones to injected calcium ions. J. Physiol. 237, 259-277.

MEECH, R.W. (1974b). Calcium influx induces a post-tetanic hyperpolarization in Aplysia neurones. Comp. Biochem. Physiol. 48A, 387-395.

MEECH, R.W. (1978). Calcium-dependent potassium activation in nervous tissues. Ann. Rev. Biophys. Bioeng., 7, 1-18.

MEECH, R.W. and STRUMWASSER, F. (1970). Intracellular calcium injection activates potassium conductance in Aplysia nerve cells. Fed. Proc. 29, 834 Abs.

MICHELL, R.H. (1975). Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta 415, 81-417.

MILLER, R.J., HORN, A.S. and IVERSEN, L.L. (1974). The action of neuroleptic drugs on dopamine-stimulated adenosine cyclic - 3',5' - monophosphate production in rat neostriatum and limbic forebrain. Mol. Pharm. 10, 759-766.

MOOLENAAR, W.H. and SPECTOR, I. (1979). The calcium action potential and a prolonged calcium dependent after-hyperpolarization in mouse neuroblastoma cells. J. Physiol. 292, 297-306.

MOORE, J.W. (1963). Operational amplifiers. In, Physical Techniques in Biological Research. Volume VI (Electrophysiological Methods), Part B, Ch. 2. Ed. Nastuk, Academic Press.

MORETON, R.B. (1972). Electrophysiology and ionic movements in the central nervous system of the snail, Helix aspersa. J. Exp. Biol. 57, 515-541.

MORITA, K., KOKETSUE, K. and KUBA, K. (1980). Oscillation of $\{Ca^{2+}\}_i$-linked $K^+$ conductance in bullfrog sympathetic ganglion cell is sensitive to intracellular anions. Nature 283, 204-205.

MURER, E.H., STEWART, G.J., RAUSCH, M.A. and DAY, H.J. (1975). Calcium ionophore A23187 (Eli Lilly). Effect on platelet function, structure and metabolism. Thrombos, Diathes. haemorrh. 34, 72-82.

NICKERSON, M. (1956). Receptor occupancy and tissue response. Nature 178, 697-698.

NISHI, S. and NORTH, R.A. (1973). Intracellular recording from the myenteric plexus of the guinea-pig ileum. J. Physiol. 231, 471-491.

NISHIYAMA, A. and PETERSEN, O.H. (1974). Membrane potential and resistance measurement in acinar cells from salivary glands in vitro: effect of acetylcholine. J. Physiol. 242, 173-188.

ORLANSKY, H. and HEIKKILA, R.E. (1974). An evaluation of various antiparkinsonian agents as releasing agents and uptake inhibitors for $^3$H-dopamine in slices of rat neostriatum. Eur. J. Pharm. 29, 284-291.

OSCHMAN, J.L. and BERRIDGE, M.J. (1970). Structural and functional aspects of salivary fluid secretion in Calliphora. Tissue & Cell 2, 281-310.

PAROD, R.J. and PUTNEY, J.W.Jr. (1978). The role of calcium in the receptor mediated control of potassium permeability in the rat lacrimal gland. J. Physiol. 281, 371-381.

PEDERSEN, G.L. and PETERSEN, O.H. (1973). Membrane potential measurement in parotid acinar cells. J. Physiol. 234, 217-227.

PETERSEN, O.H. (1978). Calcium dependence of bombesin-evoked pancreatic amylase secretion. J. Physiol. 285, 30P-31P.

PETERSEN, O.H. and IWATSUKI, N. (1978). The role of calcium in pancreatic acinar cell stimulus-secretion coupling: an electrophysiological approach. Annals New York Acad. Sci. 307, 599-615.

PETERSEN, O.H. and PEDERSEN, G.L. (1974). Membrane effects mediated by alpha- and beta-adrenoceptors in mouse parotid acinar cells. J. Membr. Biol. 16, 353-362.

PETERSEN, O.H. and UEDA, N. (1976). Pancreatic acinar cells: the role of calcium in stimulus-secretion coupling. J. Physiol. 254, 583-606.

PFEIFFER, D.R. and LARDY, H.A. (1976). Ionophore A23187: the effect of $H^+$ concentration on complex formation with divalent and monovalent cations and the demonstration of $K^+$ transport in mitochondria mediated by A23187. Biochemistry 15, 935-943.

PFEIFFER, D.R., REED, P.W. and LARDY, H.A. (1974). Ultraviolet and fluorescent spectral properties of the divalent cation ionophore A23187 and its metal ion complexes. Biochemistry 13, 4007-4014.

PHILPOTT, H.G. and PETERSEN, O.H. (1979). Extracellular but not intracellular application of peptide hormones activates pancreatic acinar cells. Nature 281, 684-688.

POSTE, G. and NICOLSON, G.L. (1976). Calcium ionophores A23187 and X537A affect cell agglutination by lectins and capping of lymphocyte surface immunoglobulins. Biochim. Biophys. Acta 426, 148-155.

POULSEN, J.H. and WILLIAMS, J.A. (1977). Effects of the calcium ionophore A23187 on pancreatic acinar cell membrane potentials and amylase release. J. Physiol. 264, 323-339.

PRESSMAN, B.C. (1973). Properties of ionophores with broad range cation selectivity. Fed. Proc. 32, 1698-1703.

PRINCE, W.T. and BERRIDGE, M.J. (1972). The effects of 5-hydroxytryptamine and cyclic AMP on the potential profile across isolated salivary glands. J. Exp. Biol. 56, 323-333.

PRINCE, W.T. and BERRIDGE, M.J. (1973). The role of calcium in the action of 5-hydroxytryptamine and cyclic AMP on salivary glands. J. Exp. Biol. 58, 367-384.

PRINCE, W.T., BERRIDGE, M.J. and RASMUSSEN, H. (1972). Role of calcium and adenosine - 3':5' - cyclic monophosphate in controlling fly salivary gland secretion. Proc. Nat. Acad. Sci. USA 69, 553-557.

PRINCE, W.T., RASMUSSEN, H. and BERRIDGE, M.J. (1973). The role of calcium in fly salivary gland secretion analyzed with the ionophore A-23187. Biochim. Biophys. Acta 329, 98-107.

PUTNEY, J.W.Jr. (1976). Biphasic modulation of potassium release in rat parotid gland by carbachol and phenylephrine. J. Pharm. Exp. Ther. 198, 375-384.

PUTNEY, J.W.,Jr. (1977). Muscarinic, alpha-adrenergic and peptide receptors regulate the same calcium influx sites in the parotid gland. J. Physiol. 268, 139-149.

REED, P.W. (1976). Effects of the divalent cation ionophore A23187 on potassium permeability of rat erythrocytes. J. Biol. Chem. 251, 3489-3494.

REED, P.W. and LARDY, H.A. (1972). A23187: a divalent cation ionophore. J. Biol. Chem. 247, 6970-6977.

REEVES, J.P. (1975). Calcium-dependent stimulation of 3-0-methylglucose uptake in rat thymocytes by the divalent cation ionophore A23187. J. Biol. Chem. 250, 9428-9430.

REUTER, H. (1974). Localization of Beta adrenergic receptors and effects of noradrenaline and cyclic nucleotides on action potentials, ionic currents and tension in mammalian cardiac muscle. J. Physiol. 242, 429-451.

REYES, A., GOICOECHEA, B. and ROSADO, A. (1978). Calcium ion requirement for rabbit spermatozoal capacitation and enhancement of fertilizing ability by ionophore A23187 and cyclic adenosine-3':5' - monophosphate. Fertil. Steril. 29, 451-455.

ROMERO, P.J. and WHITTAM, R. (1971). The control by internal calcium of membrane permeability to sodium and potassium. J. Physiol. 214, 481-507.

ROSSIGNOL, B., HARMAN, G., CHAMBAUT, A.M. and KERYER, G. (1974). The calcium ionophore A23187 as a probe for studying the role of $Ca^{2+}$ ions in the mediation of carbachol effects on rat salivary glands: protein secretion and metabolism of phospholipids and glycogen. FEBS Lett. 43, 241-246.

SCARPA, A., BALDASSARE, J. and INESI, G. (1972). The effect of calcium ionophores on fragmented sarcoplasmic reticulum. J. Gen. Physiol. 60, 735-749.

SCHLESSINGER, J., SHECHTER, Y., WILLINGHAM, M.C. and PASTAN, I. (1978). Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells. Proc. Nat. Acad. Sci. USA 75, 2659-2663.

SCHMIDT, M.J. and HILL, L.E. (1977). Effects of ergots on adenylate cyclase activity in the corpus striatum and pituitary. Life Sci. 20, 789-798.

SCHNEYER, L.H., YOUNG, J.A. and SCHNEYER, C.A. (1972). Salivary secretion of electrolytes. Physiol. Rev. 52, 720-777.

SCHREURS, V.V.A.M., SWARTS, H.G.P., DE PONT, J.J.H.H.M. and BONTING, S.L. (1976). Role of calcium in exocrine pancreatic secretion. Comparison of the effects of carbachol and the ionophore A23187 on enzyme secretion and calcium movements in rabbit pancreas. Biochim. Biophys. Acta 419, 320-330.

SCHROEDER, T.E. and STRICKLAND, D.L. (1974). Ionophore A23187, calcium and contractility in frog eggs. Exp. Cell. Res. 83, 139-142.

SELINGER, Z., EIMERL, S. and SCHRAMM, M. (1974). A calcium ionophore simulating the action of epinephrine on the α-adrenergic receptor. Proc. Nat. Acad. Sci. USA 71, 128-131.

SIEGELBAUM, S.A. and TSIEN, R.W. (1979). Calcium-activated transient outward current in cardiac Purkinje fibres. J. Physiol. 287, 36P-37P.

SMITH, R.K. and HOUSE, C.R. (1977). Fluid secretion by isolated cockroach salivary glands. Experientia 33, 1182-1183.

SMITH, R.K. and HOUSE, C.R. (1979). Ion and water transport by isolated cockroach salivary glands. J. Memb. Biol. 51, 325-346.

STEINHARDT, R.A., EPEL, D., CARROLL, E.J.,Jr. and YANAGIMACHI, R. (1974). Is calcium a universal activator for unfertilised eggs? Nature 252, 41-43.

STEPHENSON, R.P. (1956). A modification of receptor theory. Brit. J. Pharmacol. 11, 379-393.

STRADER, C.B.D., REVEL, J.-P. and RAFTERY, M.A. (1979). Demonstration of the transmembrane nature of the acetylcholine receptor by labeling with anti-receptor antibodies. J. Cell. Biol. 83, 499-510.

SUZUKI, T. and KUSANO, K. (1978). Hyperpolarizing potentials induced by Ca-mediated K-conductance increase in hamster submandibular ganglion cells. J. Neurobiol. 9, 367-392.

SWAMY, V.C., TICKU, M., TRIGGLE, C.R. and TRIGGLE, D.J. (1975). The action of the ionophores, X-537A and A-23187, on smooth muscle. Can. J. Physiol. Pharmacol. 53, 1108-1114.

TARRAB-HAZDAI, R., GEIGER, B., FUCHS, S. and AMSTERDAM, A. (1978). Localization of acetylcholine receptor in excitable membrane from the electric organ of Torpedo: evidence for exposure of receptor antigenic sites on both sides of the membrane. Proc. Nat. Acad. Sci. USA 75, 2497-2501.

THOA, N.B., COSTA, J.L., MOSS, J. and KOPIN, I.J. (1974). Mechanism of release of norepinephrine from peripheral adrenergic neurones by the calcium ionophores X537A and A23187. Life Sci. 14, 1705-1719.

THOMAS, M.V. and GORMAN, A.L.F. (1977). Internal calcium changes in a bursting pacemaker neuron measured with arsenazo III. Science 196, 531-533.

WEAKLY, J.N. (1973). The action of cobalt ions on neuromuscular transmission in the frog. J. Physiol. 234, 597-612.

WELLS, J.N., WU, Y.J., BAIRD, C.E. and HARDMAN, J.G. (1975). Phosphodiesterases from porcine coronary arteries: inhibition of separated forms by xanthines, papaverine, and cyclic nucleotides. Mol. Pharm. 11, 775-783.

WENTZELL, B. and EPAND, R.M. (1978). Stimulation of the release of prostaglandins from polymorphonuclear leukocytes by the calcium ionophore A23187. FEBS Lett. 86, 255-258.

WHITEHEAD, A.T. (1970). The innervation of the salivary gland of Periplaneta americana (L.). Amer. Zool. 10, 504.

WHITEHEAD, A.T. (1971). The innervation of the salivary gland in the American cockroach: light and electron microscopic observations. J. Morph. 135, 483-506.

WILLEY, R.B. (1961). The morphology of the stomodeal nervous system in Periplaneta americana (L.) and other Blattaria. J. Morph. 108, 219-261.

WILLIAMS, J.A. and LEE, M. (1974). Pancreatic acinar cells: use of a calcium ionophore to separate enzyme release from the earlier steps in stimulus-secretion coupling. Biochem. Biophys. Res. Commun. 60, 542-548.

WOLLHEIM, C.B., BLONDEL, B., RENOLD, A.E. and SHARP, G.W.G. (1976). Calcium induced glucagon release in monolayer culture of the endocrine pancreas. Studies with ionophore A23187. Diabetologia 12, 287-294.

WÖRNER, P. and BROSSMER, R. (1975). Platelet aggregation and the release reaction induced by ionophores for divalent ions. Thromb. Res. 6, 295-305.

WU, C.H. and NARAHASHI, T. (1976). Actions of trihexyphenidyl and benztropine on squid axon membranes. J. Pharm. Exp. Ther. 197, 135-141.

YOSHIDA, N. (1977). Actions of $Mn^{++}$ and $Co^{++}$ on the tonus and drug-induced contractions of the isolated rat rectum, particularly in relation to Ca. Kobe J. Med. Sci. 23, 41-50.

ZABUCCHI, G., SORANZO, M.R., ROSSI, F. and ROMEO, D. (1975). Exocytosis in human polymorphonuclear leukocytes induced by A23187 and calcium. FEBS Lett. 54, 44-48.

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## DECLARATION

This thesis has been composed by me and is based on work carried out by myself, either alone or in conjunction with Professor B.L. Ginsborg, Dr. C.R. House or Professor A.R. Martin.

Mary R. Mitchell

# APPENDIX

The following papers, based on work in this thesis, have been accepted for publication.

HOUSE, C.R., GINSBORG, B.L. and MITCHELL, M.R. (1978). Are dopamine receptors accessible to intracellularly applied agonist? Nature 275, 663.

GINSBORG, B.L., HOUSE, C.R. and MITCHELL, M.R. (1980). On the role of calcium in the electrical responses of cockroach salivary glands to dopamine. J. Physiol. 303, 325-335.

GINSBORG, B.L., HOUSE, C.R. and MITCHELL, M.R. (1980). A calcium-readmission response recorded from Nauphoeta salivary gland acinar cells. J. Physiol. (In Press).

MITCHELL, M.R., GINSBORG, B.L. and HOUSE, C.R. (1980). A novel effect of cobalt treatment on calcium-dependent responses of the cockroach salivary gland. Experientia 36, 98-99.

MITCHELL, M.R. and MARTIN, A.R. (1980). An electrophysiological study of the effects of ionophore A23187 on Nauphoeta salivary glands. Quart. J. Exp. Physiol. (In Press).